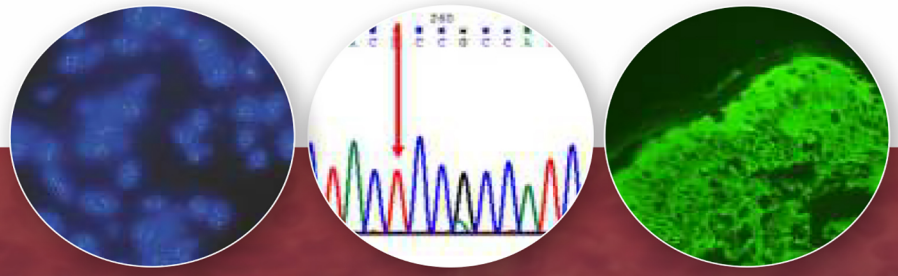
Second Edition

Seminars in Pathology



Amit Kumar Yadav

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Second Edition

Seminars in Pathology

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Seminars in Pathology

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eISBN: 978-93-546-6901-9

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Second eBook Edition: 2024

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Published by Satish Kumar Jain and produced by Varun Jain for
CBS Publishers & Distributors Pvt. Ltd.

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to

my beloved parents and my family without whose support
it would not have been possible to write this book

all the postgraduate students of pathology
for whom it has been written

all the patients who have taught me
more than textbooks

Foreword

Seminars in Pathology is a simple, lucid and easy to understand book written by a senior experienced postgraduate teacher of pathology. It is primarily intended to make the life of postgraduate students bit easier. Due to rapidly expanding knowledge they are exposed to so much information that they tend to lose focus.

I am happy to note that the first edition was very well received and read by thousands of students. The second edition will continue to guide the postgraduate students of pathology and other potential readers like senior residents and faculties in the same manner as the first edition.

For the second edition, Dr Amit Kumar Yadav has substantially updated the text wherever it was necessary. Following the advances of general pathology and systemic pathology, the revisions and additions are most evident in portions pertaining to molecular pathology and haematology. Furthermore, the author has increased the number of tables, flow diagrams and illustrations, which are so essential for student's understanding. All this has been done while maintaining the compact nature of the book.

In summary, it is my pleasure and great honor to most endorse the new second edition of this book. Dr Yadav deserves appreciation for a job well done and for providing the PG students of pathology with such an attractive and useful book. I wish the book all the success.

Reeni Malik
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Gandhi Medical College, Bhopal
Secretary
International Academy of Pathology
Indian Division (IAP-ID)

Preface to the Second Edition

The first edition of *Seminars in Pathology* was very well received and the author has been delighted to receive positive feedback from the students as well as the publisher since its publication.

The subject of pathology like its other peers in medical sciences continues to advance at a scorching pace, particularly in the field of oncopathology, with new immunohistochemical and molecular diagnostic tools transforming the way in which tumours are diagnosed and classified. This new information has been a particular focus for the updates in the second edition.

I have been aided by a number of contributors, who have been duly acknowledged throughout the book. I thank them all for their help, without which this new edition would not have been possible. I am also grateful to the team at CBS Publishers, especially Mr SK Jain and Mr YN Arjuna.

Amit Kumar Yadav

Preface to the First Edition

This book deals with recent topics of advances in various specialities of pathology. The topics are presented and organised in a manner to help the readers in easy understanding and conceptualising the knowledge so that it is retained. The format has been devised to provide maximum benefit to the postgraduate students.

The topics have been selected carefully to cover a maximum number of recent topics in current practice.

The book is expected not only to enhance understanding the subject but will also help the postgraduate students in day-to-day activity, teaching and practising pathology. It will also be easy to refresh the topics read, and thus help the students in preparing for their MD and DNB examinations.

It is hoped that the faculty will find this book extremely useful for postgraduate students of pathology and will recommend it to them.

Jasbir Singh Dhupia
Amit Kumar Yadav

Acknowledgments

I wish to acknowledge the resident doctors and staff of VMMC and associated Safdarjung Hospital who have contributed in their own way in writing the second edition of this book. I am grateful to my family for their constant support especially my two daughters Dhriti and Vartika.

I would especially like to thank my father Mr Ashok Kumar Yadav and mother Mrs Shakuntla Yadav for being the inspiration to write this book.

I would like to acknowledge all the people who are involved in the preparation of this book especially Mr SK Jain (CMD), Mr Varun Jain (Managing Director), Mr YN Arjuna (Sr Vice President—Publishing, Editorial and Publicity), Ms Ritu Chawla (GM—Production), Mr Prasenjit Paul (Editor), Mr Parmod Kumar (DTP operator), and Mr Rohan Parsad (Graphic designer), CBS Publishers & Distributors Pvt Ltd, for their continuing support to bring out this edition.

Amit Kumar Yadav

Contents

Foreword by Reeni Malik *vii*
Preface to the Second Edition *ix*
Preface to the First Edition *xi*

Section 1: GENERAL PATHOLOGY INCLUDING IMMUNOPATHOLOGY

1. Cancer Invasion and Metastasis 3
2. Dendritic Cell Migration in Health and Disease 12
3. Immune Reconstitution Inflammatory Syndrome 20
4. Principles and Clinical Utility of Measuring Immunoglobulin Subclasses 30
5. Microparticles 38
6. Recent Concepts in Tumor Immunology 45
7. Connexins and their Role in Tumour Biology 54
8. Lymphoma Microenvironment and Immunotherapy 63
9. PD-1/PD-L1 Pathway in Carcinogenesis 73
10. Techniques to Characterize Foreign Materials in Pathological Specimens 78

Section 2: RECENT ADVANCES

11. DNA Microarray 89
12. Recent Advances in Cervical Cancer Screening 97
13. Advances in Understanding of Cutaneous Mesenchymal Neoplasms 110
14. Recent Advances in Identifying Cancer Cells in Stool Samples 121
15. Pathogenesis of Ovarian Carcinoma: Recent Advances 130
16. Recent Advances in Understanding of Renal Tumors 140
17. Recent Concepts in Autoimmune Hepatitis 152
18. Advances in the Pathology of Glomerular Diseases 163
19. Autoimmune and Drug-induced Lymphadenopathies 170
20. Recent Advances in Barrett Esophagus 182
21. Molecular Pathways in Gliomagenesis 194
22. Neuroendocrine Tumors of Pancreas—Recent Advances 200
23. Recent Updates in Penile Carcinomas 211
24. Immune Therapy Related Lymphoproliferative Disorders 221

Section 3: HEMATOLOGY

25. AML Transcriptomics and Proteomics 231
26. Aplastic Anemia: Recent Concepts 243
27. Flow Cytometric Evaluation of Acute Leukemia 250
28. Inherited Platelet Disorders 263
29. Molecular Diagnosis of Thalassemia and Hemoglobinopathies 276
30. T Cell Immunotherapy for Hematological Malignancies 285
31. Targeted Therapy in Hematological Malignancies 294

Section 4: TRANSFUSION MEDICINE

32. Cord Blood Banking 307

Section 5: CLINICAL PATHOLOGY

33. Hepatitis E—Recent Advances 317
34. Laboratory Accreditation in India 331
35. Molecular Characterization of Circulating Tumor Cells 340

Section 6: HISTOPATHOLOGY

36. Approach to Vesiculobullous Disorders of Skin 353
37. Contemporary Gleason Grading of Prostatic Carcinoma 365
38. Approach to the Diagnosis of Cutaneous Lymphomas 375
39. Approach to the Diagnosis of Cutaneous Pseudolymphomas 384
40. Diagnosis and Therapy of MALT Lymphoma 396
41. Endometrial Stromal Tumor—Recent Advances 407
42. GATA3 Expression in Various Cancers 414
43. Hepatocellular Adenomas: Recent Concepts 421
44. Histopathology of Parasitic Infections of the Lung 431
45. HIV-Associated Renal Diseases 445
46. Immunohistochemistry in Diagnosis of Thyroid Tumors 455
47. Immunohistochemistry in Diagnosis of Liver Tumors 463
48. Immunohistochemistry Techniques : Basics and Recent Advances 470
49. Intraductal Papillary Mucinous Neoplasm (IPMN) of the Pancreas 480
50. Lesions of the Intermediate Trophoblast 489

51. Mesothelial Lesions: Differential Diagnosis and Diagnostic Approach 500
52. Molecular Pathogenesis of Lung Cancer 508
53. Notochord Tumors: Newer Concepts 516
54. Papillary Lesions of Breast 525
55. Pathology of Renal Transplant Rejection 533

Section 7: CYTOPATHOLOGY

56. Cell Block Preparation Techniques 547
57. Diagnostic Role of Endometrial Cell Block Cytology 552
58. Comparative Analysis of Molecular Techniques for Human Papillomavirus (HPV) in Cytology 558
59. Cytological Approach to Mediastinal Lesions 567
60. Circulating Tumor Cells (CTC): Overview and Opportunities in Cytology 578

Index 589

Section

1

General Pathology including Immunopathology

Cancer Invasion and Metastasis

1

Sakshi Sirswal

INTRODUCTION

- "Neoplasia" (*new growth* in Greek) is an abnormal proliferation of cells, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change.
- "Neoplasm"—an abnormal mass of tissue produced.
- Neoplasms can be benign, pre-malignant, or malignant (cancer).
- There are eight fundamental changes in cell physiology, which are considered the hallmarks of cancer:
  1. Self-sufficiency in growth signals
  2. Insensitivity to growth-inhibitory signals
  3. Altered cellular metabolism
  4. Evasion of apoptosis
  5. Limitless replicative potential (immortality)
  6. Sustained angiogenesis
  7. Ability to invade and metastasize
  8. Ability to evade the host immune response.

Overview of Carcinogenesis

- Carcinogenesis is a multistep process at both the phenotypic and the genetic levels, resulting from the accumulation of multiple mutations involving multiple genes.
- Phenotypically, malignancy is characterized by excessive growth, local invasiveness and ability to form distant metastases.
- The principal targets of genetic damage include:
  - Genes promoting cell growth (proto-oncogenes)
  - Genes inhibiting cell growth (tumor suppressor genes)
  - Genes regulating apoptosis
  - Genes regulating DNA repair.
- As the tumor ages, multiple mutations accumulate independently in different cells, generating subclones with varying abilities to grow, invade, metastasize, resist/respond to therapy.
- Most malignant tumors are monoclonal.

- However, by the time they become clinically evident their constituent cells are extremely heterogeneous.
- The ability of the tumor cells to metastasize is largely dependent on the ability of these cells to migrate.
- Hence an important step in carcinogenesis is epithelial-mesenchymal transformation that bestows the tumor cells with the abilities like invasion and metastasis.
- Fundamentally, the epithelial cells and mesenchymal cells have their own characteristics that allow them to behave differently.

Characteristics of Epithelial Cells

- **Epithelial cells** are cells that line the inner and outer surfaces of the body in continuous cell layers, as a single layer or multilayered structure.
- The epithelial cell layer is attached to the underlying connective tissue by a basement membrane.
- Epithelial cells are joined together by strong intercellular cell junctions that help in the maintenance of continuous cell layers.
- Epithelial cells are characterized by specialized membrane domains:
  - Basal domain
  - Lateral domain
  - Apical domain: Characteristics of this domain depend on the functional needs of the cell.
- **Lateral domain:** Keeps the epithelial layer impenetrable and consists of desmosomes, gap junctions, tight junctions and adherens junctions.
  - *Desmosomes:* Adhesion belts connected to intermediate filaments
  - *Gap junctions:* Intercellular channels 1.5–2 nm in size that permit free passage of ions and small molecules. Also, gap junctions permit changes in membrane potential.
  - *Tight junctions:* Bind cells with claudin, occludin proteins.
  - *Adherens junctions:* These are built from cadherins.
- **Basal domain:** Allows interaction between the basement membrane and the cell. The cells adhere to extracellular matrix (ECM) by:
  A. *Hemidesmosomes:* Bind the ECM to intermediate filaments via integrins.
  B. *Focal adhesions:* Bind the ECM to actin microfilaments via integrins.

Characteristics of Mesenchymal Cells

- **Mesenchymal cells** have the following characteristics:
  - They do not have cytoplasmic polarity.
  - They do not form a continuous sheet.
  - There are no specific membrane domains.
  - Intercellular adhesions are less strong which accounts for the migratory capacity of the mesenchymal cells.
  - Cells are mobile with a cytoskeleton comprised by vimentin.
  - The cells have filopodia as cytoplasmic extensions when mobile, which contain active cytoskeleton.
  - They express matrix adhesion proteins and matrix metalloproteinases (MMPs).
- Mesenchymal cells contribute to the ECM by synthesizing and organizing new components by remodeling the ECM through the production of matrix-degrading MMPs.

- Mesenchymal migration is mechanistically different from epithelial movement.
- Epithelial cells move as a sheet en block, whereas mesenchymal cells move individually.

INVASION AND METASTASIS

- Invasion and metastasis are hallmarks of malignant tumors.
- They are a major cause of cancer-related morbidity and mortality.
- The causes of metastasis can be explained using four models as follows:
  A. Metastasis is caused by rare variant clones that develop in the primary tumor.
  B. Metastasis is caused by the gene expression pattern of most cells of the primary tumor, referred to as a metastatic signature.
  C. Combination of A and B, in which metastatic variants appear in a tumor with a metastatic gene signature.
  D. Metastasis development is greatly influenced by the tumor stroma, which regulates angiogenesis, local invasiveness, and resistance to immune elimination, allowing cells of the primary tumor to become metastatic.

METASTATIC CASCADE

There are two phases of metastasis:
1. Invasion of the extracellular matrix (ECM).
2. Vascular dissemination, homing of tumor cells and colonization.

INVASION OF THE ECM

- Normally cells are glued to each other due to the presence of cell adhesion molecules, e.g. cadherins.
- Loss of E-cadherin is important in the development of almost all epithelial cancers
- The tumor cells and tumor stromal cells secrete proteolytic enzymes like—MMPs, cathepsins, urokinase and plasminogen activator.
- These proteases degrade basement membrane and interstitial connective tissue.
- Cleavage products of collagen and proteoglycans, thus formed, have chemotactic, angiogenic and growth-promoting effects.
- The loosened cancer cells bind to proteolytically generated binding sites, mainly laminin and fibronectin.
- The loss of integrins favors invasion.
- ***Locomotion*** is the final step of invasion.
- Cytokines released by tumor cells and cleavage products of matrix facilitate migration of tumor cells.

Vascular Dissemination, Homing of Tumor Cells and Colonization

- Once the cancer cells detach from neighboring cells, they migrate to surrounding tissue.
- The cancer cells then pass through blood vessels and enter the systemic circulation (intravasation).
- On reaching the metastatic site the tumor cells exit the bloodstream (extravasation).
- In this new site the tumor cells reconstitute the tissue environment which resembles that of a primary site and is favorable for the growth and survival of tumor.

- To develop migratory behavior, epithelial cancer cells acquire properties close to mesenchymal cells and this phenomenon is called epithelial-mesenchymal transition (EMT).
- EMT facilitates cell invasion and metastasis.
- However, these mesenchymal-like tumor cells gain migratory capacity at the expense of proliferative potential.
- To establish metastasis, the metastatic tumor cells undergo inverse process of mesenchymal-epithelial transition (MET).
- MET is required to regenerate a proliferative state and form macrometastases resembling the primary tumor at distant sites.

CONCEPT OF EMT

Historical Background

- Two main cell types—epithelial and mesenchymal were recognized in the late 19th century based on their shape and organization during embryonic development and interconversion between the two states was described by Frank Lillie in 1908.
- In the late 1960s, Elisabeth Hay provided a detailed description of the formation of chick primitive streak—a structure that requires the conversion of epithelial to mesenchymal cells.
- EMT was recognized as a distinct process in 1982 by the work of Garry Greenburg and Elisabeth Hay on the 3D culture of corneal epithelial cells in the laboratory.
- It took a long time for EMT to be recognized as a potential mechanism for carcinoma progression because of the following reasons:
  - EMT cannot be followed in time and space in human tumors.
  - Great diversity of cellular organization in human tumors.
  - Recognition of carcinomas (epithelial origin) and sarcomas (mesenchymal origin) as two separate entities, not thought to interconvert (except a rare tumor known as sarcomatoid carcinoma).
- The mechanisms that govern EMT are now being unraveled and many parallels are being found between EMT in embryonic development and EMT in tumor development.

Role of EMT

- Under physiological conditions, EMT plays an important role in embryonic development.
- EMT also plays a crucial role in pathological conditions like tissue reconstruction after injury (wound healing), chronic inflammation, tissue fibrosis in response to injury (lung, kidney, liver), carcinogenesis, tumor metastasis and invasion.
- However, the difference between normal development and pathological processes is at cellular and molecular level.
  - Events follow highly regulated spatial and temporal plans during physiological development.
  - During pathologic transformation, the order of events may be stochastic and time-independent or particular events may be bypassed.
- During tumorigenesis EMT may increase motility and invasiveness of cancer cells and malignant transformation may be associated with signaling pathways promoting EMT.

Epithelial Plasticity is Bidirectional

- The reverse phenomenon, back to epithelial phenotype is called, mesenchymal-epithelial transition (MET).
- MET occurs at various stages of morphogenesis, alternating with EMT during the formation of heart, somites, and kidney, and in the formation of coelomic cavities.
- ***Metastable phenotype*** refers to the ability of cells to express attributes of both epithelial and mesenchymal phenotypes.

E-CADHERIN AND EMT

- E-cadherin is a calcium-dependent transmembrane glycoprotein which functions as an adhesion molecule. It is present in most epithelial cells in embryonic and adult tissues.
- Both EMT and MET are dependent on E-cadherin.
- The cells undergoing EMT downregulate E-cadherin.
- In various human carcinomas, functional loss of E-cadherin may result from
  - Production of a defective protein
  - Promoter hypermethylation
  - Gene mutation
  - Transcriptional repression may result from the activation of repressors, such as Snail, Slug, Sip1, Ets, Twist.
- **Under physiologic conditions,** the signaling pathways that lead to cell proliferation, differentiation and migration begin with growth factor binding to a specific receptor.
- This is followed by activation of signal-transducing proteins which transmit the signal across cytosol to nucleus.
- This leads to induction and activation of nuclear regulatory factors which initiate DNA transcription.

Signaling Pathways in EMT

- Various classes of molecules that change in expression, distribution, and/or function during the EMT, and that are involved, include (Table 1.1):
  - Growth factors [e.g. transforming growth factor (TGF)-β, Wnt]
  - Transcription factors (Snails, SMAD, LEF, and nuclear β-catenin)

Table 1.1: Pathways involved in the regulation of EMT

Receptor	*Ligand*	*Signaling molecule*	*Intermediate signaling endpoint*	*Effect*
TGF-β receptor	TGF-β	RhoA	MRTF-A	Stress fibers migration
FGF receptor	FGF	Smad	SNAIL2	Cytoskeleton activation migration, focal adhesion, rearrangement
Tyrosine kinase	HGF	Sarc	SNAIL2	Increased migration
Integrins	EGF	Ras, MAPK	SNAIL1	E-cadherin downregulated, reduced cell adhesion
Frizzled	Collagen, fibronectin	Paxillin, Rac	GIT1	Stress fibers migration

- Molecules of the cell-to-cell adhesion axis (cadherins, catenins)
- Cell-to-ECM adhesion axis (integrins, focal contact proteins, ECM proteins)
- Cytoskeletal modulators (Rho family)
- Extracellular proteases (matrix metalloproteinases, plasminogen activators).
- Other molecular changes seem to occur after the initial behavioral change; for example, there is often a trend to replace cytokeratin intermediate filaments with other types, typically vimentin.

INDUCTION OF EMT

- The first event in EMT is the proteolytic digestion of the basement membrane by metalloproteinases.
- Local expression of TGF-β, EGF, IGF-II and FGF-2 facilitates EMT by binding to receptor with ligand-inducible intrinsic kinase activity.
- Overexpression of master regulators of EMT, such as the transcription factors like Twist, Snail, and SIP1 repress the expression of E-cadherin.

TGF-β Pathway or Smad Signaling Pathway

- TGF-β interacts sequentially with two membrane receptors.
- TGF binds first to the type II receptor and then the ligand-receptor complex associates with type I TGF receptor.
- TβIIR phosphorylates TβIR.
- Activated TGF-RI propagates the signal downstream by phosphorylating Smad2 and Smad3 which form complexes with Smad4 and translocate into the nucleus.
- In combination with T cell factor (TCF) family transcription factors, they down-regulate E-cadherin genes and initiate EMT.

Wnt/β-catenin Signaling Pathway

- Wnt signals through a family of cell surface receptors called frizzled (Frz).
- It stimulates several pathways, the central one involves **β-catenin** and APC.
- It regulates the amounts of β-catenin protein available within the cell for binding cadherins.
- It mediates
  - cell-cell adhesion
  - adhesion to cytoskeletal (F actin) elements
- In resting cells, β catenin forms complex containing glycogen synthase kinase3 (GSK3β), axin and APC protein.
- Phosphorylated β-catenin is degraded by ubiquitination.
- Hence, intracellular level of β-catenin is kept low.
- Wnt-Frz leads to dissociation and inactivation of GSK3β, which can no longer phosphorylate β-catenin.
- Free β-catenin translocates to the nucleus, thereby inducing gene expression in a complex with T cell factor (TCF) down-regulating E-cadherin and initiating EMT.
- For example, in APC-mutated in colon cancer, cells behave as if they are under constant stimulation by Wnt pathway.
- This leads to EMT induction as β-catenin translocates to nucleus.

Tyrosine Kinases Pathway

- Several growth factors can induce EMT by binding to receptor tyrosine kinases.
- FGF, EGF, TGF-α and IGF-2 can induce EMT.

TRANSCRIPTION FACTORS ASSOCIATED WITH EMT

Twist

- Twist transcription factors cooperate with mitogenic oncoproteins in cancer cells.
- Twist contributes to the malignant transformation via the following:
  - Overrides premature senescence (inhibits apoptosis).
  - Downregulates E-cadherin.
  - Upregulates mesenchymal markers like Vimentin, SMA.
  - Induces EMT allowing tumor progression and dissemination.

Snail

- Snail is a strong direct repressor of E-cadherin.
- It confers tumorigenic, invasive and migratory properties.
- It inhibits apoptosis.
- It inversely correlates with the degree of differentiation and is associated with lymph node metastasis.

Steroid Receptor Coactivators Interacting Protein (SIP)

- SIP, a novel ankyrin repeat containing protein, sequesters steroid receptor coactivators in the cytoplasm.
- It contains a Smad-binding domain and may therefore modulate TGF signaling pathway, which is known to induce EMT.

EMT MARKERS

- Table 1.2 gives a list of markers that can be used for diagnosing EMT.

Table 1.2: Markers for EMT

Proteins that increase in abundance	
N-cadherin	MMP-2
Vimentin	MMP-3
Fibronectin	MMP-9
Snail1 (Snail)	Integrin αvβ6
Snail2 (Slug)	N-cadherin
Twist	Vimentin
FOXC2	Fibronectin
Sox10	Snail1 (Snail)
Proteins that decrease in abundance	
E-cadherin	Cytokeratin
Desmoplakin	Occludin

Contd.

Table 1.2: Markers for EMT	
Proteins that decrease in abundance	
β-catenin	Snail1 (Snail)
Smad-2/3	Snail2 (Slug)
NF-κβ	Twist
	β-catenin
Proteins whose activity increases	
ILK	Rho
GSK-3β	
***In-vitro* functional markers**	
Increased migration	Increased scattering
Increased invasion	Altered cell shape

EMT in Cancer

- Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion and migration capacity.
- Thus there is a derangement of apicobasal polarity and cell-to-cell adhesive architecture and function, lack of basal lamina integrity.
- The occurrence of EMT during tumor progression allows tumor cells (i.e., ones that are noninvasive and nonmetastatic) to acquire the capacity to infiltrate surrounding tissue and ultimately metastasize to distant sites.
- The most compelling evidence for the involvement of EMT in oncogenesis is the ability of multiple EMT regulators to enhance tumor formation and/or metastasis.

Studies to Prove Role of EMT in Cancer

- For example, expression of Snail1 increases the aggressiveness of experimentally induced breast tumors, and high Snail1 expression correlates with an increased risk of tumor relapse and poor survival rates in human breast cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma, and proteomic analysis of breast cancer reveals that circulating mammary tumor cells, or those found as micrometastases, show evidence of mesenchymal conversion.
- Nuclear localization of β-catenin is frequently used as an EMT marker, and nuclear β-catenin is a marker for a poor prognosis in colorectal cancer.

THERAPEUTIC IMPLICATIONS

- Strategies to block any steps during EMT would have a major impact on EMT and, thereby, on fibrosis, and cancer metastasis.
- Blockade of growth factor, cytokine and integrin pathways that lead to activation of signaling elements such as β-catenin and transcription factors Slug, Snail, Twist which assume critical roles in EMT may constitute potent strategies to counteract the progression from localized cancers to disseminated disease.

- EMT-associated molecules can be used as markers for the prediction of prognosis and response to targeted therapy.
- Commonly used molecular markers for EMT include increased expression of N-cadherin, vimentin, and integrins.
- IHC may be used to detect the expression of these molecular markers. IHC of EMT involved molecules may also be used to predict metastatic behavior.
- Nuclear localization of β-catenin is frequently used as an EMT marker, and nuclear β-catenin is a marker for a poor prognosis in colorectal cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma.
- Blockade of EGFR signaling pathway by using anti-EGFR antibody or EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib inhibit invasion.
- BMP-7 antagonizes TGF-β driven EMT in fibrotic kidney and heart inhibit disease development.

POINTS TO REMEMBER

- Accumulating evidence in recent years indicates that EMT is a critical process not only in development but also in tumorigenesis.
- Acquisition of EMT properties during tumor progression is associated with dissolution of epithelial integrity, increased migration, local invasion and, ultimately, metastasis.
- For its association with invasion and early steps of metastasis, inhibition of EMT appears a viable strategy for novel approaches to cancer therapy.
- However, given the complex, intertwined circuitry regulating EMT, effective deployment of anti-EMT therapeutics cannot be done without understanding the molecular alterations of the specific tumor being targeted.

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2 Dendritic Cell Migration in Health and Disease

INTRODUCTION

- Dendritic cells (DCs) were discovered by Ralph Steinman in 1973.
- They are a component of the innate immune system.
- Their main function is to process antigen material and present it on the surface to T cells, thus functioning as antigen-presenting cells.

Origin of Dendritic Cells

Location

They are seen in the following locations:

1. Skin: Langerhans cells in the epidermis, dermal dendritic cells.
2. Under epithelia of lungs, stomach and intestine.
3. Immature state in the blood.

Functions of Dendritic Cells

- Antigen presentation and activation of T cells.
- Induction and maintenance of immune tolerance.
- Maintaining immune memory in tandem with B cells.

Subsets of Dendritic Cells

- There are three main subsets as follows:
  1. Plasmacytoid dendritic cell (pDC)
  2. Conventional DC1 (cDC1)
  3. Conventional DC2 (cDC2)
- Minor subsets include—Monocyte-derived DC (MoDC) and double negative DCs.
- The immunological profile of cDC1 and cDC2 varies according to the various sites.

DC Migration Routes

Maturation and Mobilization of Tissue Resident DCs

- During embryonic development, as well as postnatally, DC progenitors populate the skin, mucosal surfaces and most solid organs of the body to give rise to immature classical dendritic cells (cDCs).

- These act as sentinels of the immune system.
- They display characteristic dynamic stellate-like extensions.
- These extensions allow these cells to exhibit sweeping movements and to engage in multiple contacts with each other.
- This leads to the formation of a dense cellular network for the efficient detection of invading pathogens.
- Immature cDCs are rather immotile and are specialized in the sampling of foreign as well as self-antigens.
- They use numerous mechanisms of antigen acquisition, including receptor-dependent endocytosis and macropinocytosis.
- Subsequently, they undergo activation.
- This is either triggered by an intrinsic program or due to the recognition of molecules associated with pathogens and microbiota.
- Subsequently an increase in motility corresponding to upregulation of CC-chemokine receptor 7 (CCR7) is seen.
- CCR7 interacts with its ligands CC-chemokine ligand 19 (CCL19) and CC-chemokine ligand 21 (CCL21).
- They are found on lymphatic endothelial cells.
- They guide DCs towards and into the lumen of initial lymphatic vessels (also known as 'terminal lymphatics').
- This process is known as haptotaxis.
- Haptotaxis is defined as persistent directional migration along gradients of a chemoattractant that are immobilized on cells and/or elements of the extracellular matrix.
- The terminal blind-ended lymphatic sacs are equipped with a discontinuous basement membrane and flap valves.
- These allow DC entry without pericellular proteolysis.

Intranodal Migration and Positioning of Dendritic Cells

- Once inside the afferent lymphatic vessel, DCs first crawl along the vessel wall but are then passively transported by the lymph flow into the subcapsular sinus (SCS) of lymph nodes.
- Subsequently they enter the lymph node parenchyma through the SCS floor.
- Atypical chemokine receptor 4 (ACKR4) is specifically expressed by lymphatic endothelial cells (LECs) lining the ceiling, but not the floor.
- This causes a CCL21 chemokine gradient that points towards the lymph node paracortex.
- This supports the efficient translocation of lymph-derived DCs into the T cell rich area.
- After their entry from afferent lymph, migratory DCs join the network of the largely sessile lymph node-resident DCs.
- These are embedded in the stromal network of the T cell areas and acquire antigen from the so-called lymph node conduits.
- Pre-cDCs engage L-selectin to gain access to lymph nodes via high endothelial venules (HEVs).

- Plasmacytoid dendritic cells (pDCs) also primarily enter lymph nodes via HEV.
- CCR7 again is responsible.

DC Migration in the Skin

DC Subsets in the Skin

- Langerhans cells (LCs), expressing C-type lectin langerin (CD207) are the only tissue resident DC population in the epidermis.
- Dermal DCs comprise several subsets including XC-chemokine receptor 1 (XCR1)+ conventional DC1s (cDC1s), CD11b+ conventional DC2s (cDC2s), and XCR1-CD11b- double negative cDCs
- In addition, and especially during inflammation, CCR2+ monocyte-derived DCs (moDCs) are recruited to the dermis.
- Like cDC2s, moDCs express CD11b.

LCs in the Epidermis

- LCs arise from yolk sac-derived erythromyeloid progenitors (EMPs).
- These cells populate the fetal liver and give rise to an initial wave essentially all tissue resident macrophages as well as skin LCs.
- These cells regenerate from local precursors throughout adult life.
- Transforming growth factor-β1 (TGF-β1) from both autocrine and paracrine sources as well as keratinocyte-derived interleukin 34 (IL-34) are required for the maintenance and homeostatic self-replenishment of LCs.
- In the noninflamed skin, LCs are nonmotile and 'anchored' to keratinocytes by E-cadherin-containing tight junctions.
- Also, TGF-β1 inhibits steady-state and inflammation-induced motility of LCs.
- Following activation in the skin, LCs reach the draining lymph node later than dermal DCs and populate different areas.
- LCs migrate into the deep T cell zone, dermal DCs reside at interfollicular areas and colonize regions directly beneath the B cell follicles.
- Recent evidence suggests that LCs promote *peripheral tolerance induction*.
- By migrating to cutaneous lymph nodes, LCs mediate deletional tolerance in CD4+ as well as CD8+ T cells, induce regulatory T (Treg) cell development and promote the production of immunosuppressive IL-10
- Immigration of (bone marrow-derived) blood-borne cDC precursors (pre-cDCs), plasmacytoid DCs (pDCs), monocytes and circulating (immature) cDCs into different mucosal and non-mucosal peripheral tissues via resting or inflamed postcapillary venules.

Dermal cDCs

- All dermal cDCs are derived from hematopoietic stem cell (HSC) derived progenitor cDCs (precDCs).
- These cells continuously repopulate the adult dermis.
- Recent evidence suggests that different precDC subsets exist in the adult bone marrow and blood that are already committed to the cDC1 or cDC2 lineage.
- Dermal cDC1s efficiently cross-present antigen.

- Dermal cDC1s carry antigens derived from the skin commensal *Staphylococcus epidermidis* to cutaneous lymph nodes and induce *S. epidermidis* specific IL-17-producing CD8+ T cells that home back to the skin.
- There, these commensal-specific T cells get further activated by IL-1 that is secreted by CD11b+ skin resident DCs (either cDC2s or moDCs).
- T cells generated by this 'division of labor' between different subsets of skin DCs are thought to contribute to antipathogen defense of keratinocytes in an IL-17 dependent manner.
- cDC2s can respond to thymic stromal lymphopoietin (TSLP) expressed by keratinocytes.
- It is strongly elevated in skin lesions of patients with atopic dermatitis.

MIGRATORY DCs OF THE INTESTINES

- Migration of intestinal DCs has an important role in the following
  - Immune surveillance and homeostasis of the gut
  - Maintenance of tolerance towards commensals and food proteins.
- Migratory intestinal DCs can be derived from three different sites:
  1. Lamina propria
  2. Peyer's patches
  3. Solitary intestinal lymphoid tissues (SILT).

Lamina Propria DCs

- Lamina propria contains (at least) three distinct populations of cDCs
  - Intestinal cDC1s: Characterized by expression of CD103 and lack of CD11b.
  - Intestinal cDC2s: CD103+CD11b+ .
  - Double negative DCs: CD103–CD11b-.
- Lamina propria-derived CD103+ cDCs in general, exhibit a special propensity to induce regulatory T (T-reg) cells.

Antigen Uptake in the Gut

- CD103+CD11b+ intestinal DCs are recruited from the lamina propria into the epithelial cell layer.
- These intraepithelial DCs are the first to acquire any antigen.

Intestinal pDCs Mobilize cDCs

- The pDCs themselves do not migrate to the draining mesenteric lymph nodes.
- However, they do mobilize lamina propria cDCs to migrate to mesenteric lymph nodes in a TLR- and tumor necrosis factor (TNF)-dependent manner.

DC Migration and Immune Responses to Commensals

- Intestinal DCs carry live commensals to the mesenteric lymph nodes.
- This triggers the production of secretory IgA, which subsequently helps to prevent mucosal penetration by commensals.
- Sampling of commensals by DCs drives mucosal TH17 cell differentiation.
- This occurs locally in the gut and does not rely on the migration of antigen-presenting cells to secondary lymphoid organs.

DC Migration During Intestinal Infection

- Acute intestinal infection can lead to tissue damage including leakage of lymph vessels.
- Accumulation of migratory DCs occurs in the surrounding mesenteric adipose tissue.
- If this DCs migration to the mesenteric lymph nodes is prevented, it results in compromised mucosal immune responses.
- This is the likely reason why persistent infections, gut inflammation, and environmental enteropathy cause weak performance of oral vaccines.

MIGRATION OF LUNG-DERIVED DCs

- Lung DCs migration to mediastinal lymph nodes depends on CCR7 and complement-mediated signaling.
- FTY720 and prostaglandin D2 suppress respiratory DC migration to mediastinal lymph nodes.
- Age-dependent increases in prostaglandin D2 levels result in impaired DC migration and reduced T cell responses.
- This is linked to poorer clinical outcomes in older patients with severe respiratory viral infections.

DC Migration and Asthma

- Inflammation during allergen challenge did not significantly alter DC motility.
- However, it was associated with accumulation of CD11b+ DCs near airways.
- This depends on CCR6 expression on DCs and fibroblast expression of the integrin $\alpha v\beta 8$ (a critical activator of TGFβ).
- $\alpha v\beta 8$-mediated TGFβ activation is known to enhance IL-1β-dependent fibroblast expression of the CCR6 ligand, CCL20.

DC Migration During Respiratory Infection

- In respiratory virus infection, migration of lung DCs to mediastinal lymph nodes is required for the generation of cytotoxic T cell responses.

Infection by Influenza Virus

- In the early post-infection phase CD103+ cDCs are the predominant lung DC population transporting influenza virus to the mediastinal lymph nodes.
- At a later stage CD11b+ DCs accumulate in the mediastinal lymph nodes and dominate CD8+ T cell stimulation.

Mycobacterium Tuberculosis

- Migration of lung DCs to mediastinal lymph nodes after *Mycobacterium tuberculosis* exposure requires IL-12p40.
- Gene ASAP1, encoding a regulator of DC migration, is associated with susceptibility to tuberculosis.
- Genetically determined excessive reduction of ASAP1 expression impairs migration of *M. tuberculosis*-infected DCs and may delay the onset of adaptive immunity.

DC MIGRATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

- Cerebrospinal fluid has a role in excretion, physical protection and immunological function, allowing for the transport of CNS-derived soluble antigens and immune cells.
- CNS parenchyma is largely devoid of DCs under steady-state conditions.
- DCs primarily reside within the choroid plexus and the meninges of the non-injured, steady-state CNS.
- Macrophages reside in various CNS compartments under steady-state conditions, including:
  - Parenchymal microglia
  - Perivascular
  - Meningeal
  - Plexus macrophages.
- During CNS inflammation, there is an additional increase in monocyte-derived macrophages.
- Macrophages are thought to be rather non-migratory, primarily fulfilling *in situ* functions such as tissue homeostasis or local lymphocyte stimulation.

DC MIGRATION IN INFLAMMATORY DISEASES

Rheumatoid Arthritis

- Both immature and activated DC populations are found within the inflamed joint synovium in rheumatoid arthritis.
- Pannus (Representing ectopic lymphoid tissues) developing in rheumatoid arthritis allows for the entry of circulating pre-cDCs and/or cDCs from the blood by means of chemokine signals.
- These include CX3CR1, CCR9, CXCR4.

Systemic Lupus Erythematosus

- pDCs play a crucial role in systemic lupus erythematosus (SLE), especially in the early phases of the disease.
- Number of circulating pDCs within the blood of SLE patients is markedly reduced, and this correlates with disease activity.
- Thus, it is quite plausible that the recruitment of pDCs into lymph nodes and/or into diseased tissues, such as the skin, might contribute to SLE progression.
- SLE serum factors, in particular, high levels of type I interferon could synergize with bacterial lipopolysaccharide.
- They induce the differentiation of a subset of monocytes into CCR7-expressing migratory moDCs.
- This could explain the commonly observed phenomenon of SLE 'flares' triggered by infections.

Psoriasis

- Psoriasis is an inflammatory disorder of the skin, characterized by a massive infiltration of effector cells including the following:
  - Natural killer (NK) cells
  - Type 3 innate lymphoid cells (ILC3s)
  - TH1 cells
  - TH17 cells and γδ T cells, that drive the inflammatory process by producing IL-17 and IL-22.
- Keratinocytes themselves, produce IL-36 that stimulates dermal cDCs to produce IL-23, which in turn drives TH17 cell activation.

General Concepts of DC Migration

- Any antigen from the peripheral tissues can reach to lymph nodes in two ways:
  1. Using DCs
  2. As free soluble Ag
- The first method has certain distinct advantages. These are as follows:
  - Delivery of an adequate amount of antigen to lymph nodes is ensured. This leads to the induction of an appropriate immune response. For example, intracellular or tightly cell-bound antigens.
  - Retention of arriving DCs in draining lymph nodes helps to effectively 'concentrate' rare antigens within the lymph nodes, thereby enhancing antigen presentation to T cells.
  - Compartmentalization of immunity facilitates tissue-specific responses. For example, immune responses towards commensals are restricted to mucosal immune compartments and do not induce potentially detrimental systemic inflammatory reactions.

POINTS TO REMEMBER

- The migration from non-lymphoid to lymphoid tissue is a key feature of DCs.
- CCR7 is key in facilitating this process in generally all tissues.
- In steady-state conditions DC migration is essential for the induction of peripheral tolerance.
- The relative importance of migratory DCs for mounting protective immunity during infections seems to depend on both the pathogen itself and the tissue site affected.
- Mechanisms that allow precDCs to populate nonlymphoid organs during development and replenishment are not understood.
- Therapeutic approaches that target the migratory machinery of DCs possess an enormous potential for interfering with unwanted immune responses.
- In this regard strategies that target CCR7 or other key mediators of DC trafficking may represent promising novel approaches to target DCs in the clinic.

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3 Immune Reconstitution Inflammatory Syndrome

Anam Singh

INTRODUCTION

- Immune reconstitution inflammatory syndrome (IRIS) is a paradoxical clinical worsening of a known condition or the appearance of a new condition after initiating antiretroviral therapy (ART) in HIV-infected patients.
- Since clinical deterioration occurs during immune recovery, this phenomenon has been also described as immune restoration disease (IRD)/immune reconstitution syndrome (IRS)/paradoxical reactions.
- Antiretroviral therapy in HIV/AIDS patients leads to dramatic reductions in plasma viral load, improvement in CD4+ T cell counts and partial restoration of overall immune function.
- These immunological changes correlate with reduction in the frequency of opportunistic infections (OI) and prolonged survival.

HISTORY

- This condition was first noted following the introduction of Zidovudine monotherapy in the early 1990s.
- In these cases localized forms of *Mycobacterium avium*-intracellulare (MAI) infection were observed in association with the recovery rather than failure of cellular immune responses.

Epidemiology

- Despite numerous descriptions of the infectious and non-infectious causes of IRIS, the overall incidence of the syndrome itself remains largely unknown.
- However, it is estimated that 17–23% of patients initiating ART will develop the syndrome.
- The variation in reported frequency reflect differences in case definitions, and more importantly, differences in study populations with differing risk profiles and underlying burden of opportunistic infections.
- Most of the literature on epidemiology comes from the developed countries.
- In a series from southern India TB-IRIS was reported in 7.6% of patients receiving ART.

- It is expected that IRIS will become more common in resource-constrained settings like India, where access to ART is increasing.
- The underlying prevalence of opportunistic infections (OI) like *M. tuberculosis* is high in this setting and patients initiating ART are more likely to have advanced immune-suppression.
- Although IRIS is now a well-established entity, uncertainty exists with regards to its pathogenesis and management.
- Research in the field is hampered by lack of a consistent definition of the syndrome.

Risk Factors

- Male sex and younger age.
- Lower CD4+ cell count at ART initiation (especially <50 cells/μl).
- Higher HIV RNA at ART initiation.
- Lower CD4:CD8 ratio at ART initiation.
- More rapid initial fall in HIV RNA on ART.
- Shorter interval between OI therapy initiation and ART initiation.
- Disseminated versus localized OI.
- ART-naïve patient.

Diagnostic Criteria

- General IRIS case definition was proposed by ***French et al*** (2004).
- Diagnosis requires two major criteria or one major criterion plus two minor criteria to be fulfilled.

Major Criteria

1. Atypical presentation of opportunistic infections or tumors in patients responding to ART, manifested by any of the following:
   - Localized disease.
   - Exaggerated inflammatory reaction.
   - Atypical inflammatory response in affected tissues.
   - Progression of organ dysfunction or enlargement of pre-existing lesions after definite clinical improvement with pathogen-specific therapy prior to ART and exclusion of treatment toxicity and new diagnoses.
2. Decrease in plasma HIV RNA level >1 log10 copies/mL.

Minor Criteria

1. Increased blood CD4+ T cell count after ART.
2. Increase in immune response specific to the relevant pathogen, e.g. delayed hypersensitivity response to mycobacterial antigens.
3. Spontaneous resolution of disease without specific antimicrobial therapy or tumor chemotherapy with continuation of ART.
4. ***Robertson et al (2006)*** also gave their criteria. These are divided into required and supportive criteria.

Required criteria

- Worsening symptoms of inflammation/infection.
- Temporal relationship with starting of antiretroviral treatment (3 months).

- Symptoms not explained by newly acquired infection or disease or the usual course of a previously acquired disease.
- >1 log10 decrease in plasma HIV load.

Supportive criteria

- Increase in CD4+ cell count of ≥25 cells/μl.
- Biopsy demonstrating well-formed granulomatous inflammation or unusually exuberant inflammatory response.

Pathogenesis

- The immunopathology of IRIS is determined by the inciting pathogen.
- Both CD4+ and CD8+ effector T cells are involved in the pathogenesis.
- The syndrome appears to result due to an unbalanced immune reconstitution of effector (Pro-inflammatory Th17) and regulatory T cells in patients receiving ART.
- Macrophages and natural killer cells are also suspected to play a role in IRIS.
- Due to inappropriate activation of macrophages TB-IRIS develops.
- NK cells are responsible for herpes IRIS.
- Whether elicited by an infectious or non-infectious agent, the presence of an antigenic stimulus for development of the syndrome appears necessary.

Types of IRIS

IRIS is of 2 types:

1. Unmasking IRIS
2. Paradoxical IRIS

Unmasking IRIS

- It is defined as immune response against a pathogen that was not causing overt clinical disease before initiation of ART.
- This type of IRIS is characterized by atypical exuberant inflammation and/or an accelerated clinical presentation suggesting a restoration of antigen-specific immunity.
- When infectious it is the antigenic response to pathogenic infection, and when non-infectious it is due to antigenic response to self-antigen.
- This type of IRIS usually presents during the first 3 months of therapy.
- Viable pathogens may be isolated from samples obtained from affected body sites.
- Diagnostic criteria.
- Clinical criteria:
  - Temporal relationship: ART initiation must precede clinical deterioration.
  - New onset of symptoms of an infectious or inflammatory condition after initiation of ART.
  - Consistent with the presence of pre-existing causative pathogen or antigen at the time of starting ART.
  - Either of the following:
    - Onset within 3 months after initiating ART.
    - Atypical or exaggerated clinical, histological or radiological findings in terms of severity, character of inflammatory response, rapidity of onset or localization.

- Exclusion of other causes:
  - Event not explained by:
    - Expected clinical course of another condition.
    - Drug toxicity.
    - Newly acquired infection, based on clinical or other evidence.
    - Failure of antiretroviral therapy: Presumptive based on nonadherence or resistance to ART, or confirmed based on VL assay, if available.

Paradoxical IRIS

- It occurs when the opportunistic infection is present at initiation of ART which worsens on therapy.
- This may be a response to living pathogens or a response to the antigens of non-viable pathogens.
- This form is also most common during the first 3 months of therapy but may also present later**.**
- IRIS is precipitated by the degree of immune restoration following ART.
- An alternative immunological mechanism may involve qualitative changes in lymphocyte function or lymphocyte phenotypic expression.
- Following ART, there is restoration of immune response with redistribution of T cells from peripheral lymphoid tissue, causing increased memory CD4 cells.
- After this redistribution, naïve T cells increase and are thought to be responsible for the later quantitative increase in CD4+ cell counts.
- Thus, IRIS may be due to a combination of both quantitative restoration of immunity as well as alterations in qualitative function and phenotypic expression observed soon after the initiation of ART.
- Clinical criteria
  - Temporal relationship: ART initiation must precede clinical deterioration.
  - One of the following:
    - Worsening of an infectious or inflammatory condition that was recognized and ongoing at the time of ART initiation, following an appropriate response to clinical treatment.
    - Deterioration with atypical or exaggerated clinical, histological or radiological findings in terms of severity, character of inflammatory response, rapidity of onset or localization.
    - Recurrence of an episodic infectious or inflammatory condition, worse than episodes within 1 year preceding ART in terms of frequency, severity or response to therapy.
- Exclusion of other causes
  - Worsening not explained by:
    - Expected clinical course of underlying condition, given current therapy with the susceptibility profile of the organism.
    - Drug toxicity.
    - Other infection or inflammatory condition.
    - Withdrawal of previous effective therapy.
    - Failure of antiretroviral therapy: Presumptive based on nonadherence or resistance to ART, or confirmed based on VL assay, if available.

Biomarkers of IRIS

- Various inflammatory markers, chemokines and cytokines that signify innate and adaptive immune activation may act as useful biomarkers.
- The role of these biomarkers has been assessed mainly in TB-IRIS and cryptococcal meningitis (CM)-IRIS.
- These biomarkers include:
  - C-reactive protein (CRP)
  - Interferon (INF)-γ
  - Interleukin (IL)-2, 6, 7, 12, 13, 17, 18
  - Tumor necrosis factor (TNF)-α
  - INF-γ inducible protein-10 (IP-10)
  - D-dimer.

DISEASE SPECIFIC-IRIS

Mycobacterium tuberculosis IRIS

- *Mycobacterium tuberculosis* (TB) is among the most frequently reported pathogen associated with IRIS.
- Incidence varies from 8 to 43%.
- The incidence of IRIS is expected to rise in this patient group because of the wide availability of HAART in India now.

Risk Factors

- These include the following:
  - Paradoxical TB-IRIS
  - Shorter delay between commencing ATT and ART
  - Low baseline CD4+ cell count
  - Higher baseline viral load
  - Rapid reduction in viral load
  - Disseminated tuberculosis
- Majority of the cases develop IRIS within the initial 2 months of ART when the antigen burden is high.

Pathogenesis

- Recirculation of previously sequestered CD45RO memory lymphocytes allows the pathogen-specific cells to gain access to the sites of infection and mount an inflammatory response.
- In normal condition, when Mycobacterium infection acts on resting macrophage, CD4 cells produce IFN gamma to activate the macrophage to achieve bacterial containment.
- In case of HIV, there are a few or no CD4 cells and when Mycobacterium infection acts on resting macrophage, then it leads to formation of primed macrophages which get accumulated.
- When the CD4 cells are restored post-ART, accumulated primed macrophages produce increased inflammatory cytokines causing excessive inflammation and tissue destruction.

Case Definition for Tuberculosis-associated IRIS

- This was given by ***Colebunders et al***, 2006.
- **Suspected case** (all 3 should be met)
  - An initial clinical response to ATT.
  - New persistent fevers without another identifiable cause and/or one or more of the following:
    - Worsening or emergence of dyspnea
    - Stridor
    - Increase in lymph node size, development of abscesses
    - Development of abdominal pain with ultrasound evidence of abdominal lymphadenopathies
    - Unexplained CNS symptoms.
  - Adequate adherence to ART and tuberculosis treatment
- **Confirmed case** (must meet the following three criteria)
  - New/worsening radiological signs.
  - A good virological response and/or increase in CD4+ lymphocyte count, and/or conversion of tuberculin skin test from negative to positive
  - Adequate adherence to ART and ATT.
  - Exclusion of treatment failure or other concomitant infections, tumors, or allergic reactions.

Treatment

- Treatment for mycobacterial-associated IRIS depends on the presentation and disease severity.
- Most patients present with non-life-threatening presentations which respond to the institution of appropriate ATT.
- Treatment can also be done with NSAIDs and severe cases with corticosteroids.

Atypical Mycobacteria

- Atypical mycobacteria are also frequently reported as causative pathogens in IRIS.
- MAC-associated IRIS typically develops in severely immunosuppressed individuals, who have an excellent response to ART.
- The syndrome manifests usually after 2–8 weeks of ART in patients with low CD4 counts.

Clinical Features

- Fever
- Suppurative painful lymphadenitis
- Followed by pulmonary disease
- May also involve joints, spine, skin and soft tissue
- In contrast to disseminated MAC disease of advanced AIDS, MAC IRIS usually presents as localized disease.
- Treatment is similar to TB-IRIS.
- Occasionally, surgical excision of profoundly enlarged nodes or debridement of necrotic areas has been reported.

Cytomegalovirus (CMV) Infection

- CMV retinitis may be seen either in patients with a prior history of CMV retinitis or in patients with no previous evidence of retinitis.
- It is speculated that an ART-induced inflammatory response may be responsible for unmasking a subclinical infection.
- This is seen exclusively in people with previous CMV retinitis infection who responded to ART therapy (paradoxical).
- In addition to classical CMV retinitis, ART leads to new clinical manifestations of the infection, termed immune recovery vitritis (IRV) and immune recovery uveitis (IRU).

Immune recovery vitritis

- IRV presents with acute onset of blurred vision and "floaters" caused by posterior segment inflammation.
- Ophthalmologic examination reveals numerous inflammatory cells in the vitreous humor.
- Symptoms usually resolve in one month without specific treatment and without any lasting visual effects.

Immune recovery uveitis

- IRU may occur within months of ART initiation, but typically is a late complication; occurring about 3 years after patients begin ART.
- It often results in macular edema, epiretinal membrane formation, and/or cataracts, which can lead to permanent vision loss and hence requires a high index of suspicion.

Treatment

- Anti-CMV therapy with gancyclovir or valgancyclovir
- However, IRU may not respond to anti-CMV therapy
- The use of systemic corticosteroids has been successful, and IRV may require peri-ocular corticosteroid injections.

Varicella Zoster Virus Infection

- With the introduction of protease inhibitors (PI), increasing rates of herpes zoster were noted in HIV-infected patients.
- Incidence rates are three to five times higher than those observed in the pre-HAART era.
- Mean onset of disease from ART initiation is 5 weeks (range 1–17 weeks).

Pathogenesis

- Normally, CD8 cells are implicated in the reactivation of varicella zoster infection in non-HIV patients.
- Therapy with PI causes an increase in CD4 as well as CD8 cells.
- Most common presentation is typical dermatomal involvement without dissemination or systemic symptoms.
- Although complications such as encephalitis, myelitis, cranial and peripheral nerve palsies, and acute retinal necrosis can also occur.

Cryptococcus Neoformans (CN) IRIS

- Paradoxical CN-IRIS incidence has been reported to be 10–42%, in ART naïve patients with *Cryptococcus.*
- Approximately 60% of cases occur within the first month, although the symptoms may present as late as 10 to 12 months after treatment.

Clinical Features

- Meningitis, lymphadenitis, pneumonitis, and localized abscess.
- Overall incidence of neurological IRIS is 1.5% in individuals with CD4+ counts <200 cells/μl.
- The majority of cryptococcal IRIS cases represent reactivation of previously treated cases, suggesting either an immunological reaction to incompletely treated disease or an inflammatory reaction to residual antigens.
- Unlike many other forms of IRIS, which produce less dramatic consequences, CM-IRIS is exceptional for its substantial morbidity and mortality.

Treatment

It has three phases:

1. Induction phase for 14 days with amphotericin B
2. Consolidation phase with fluconazole for 8 weeks
3. Suppressive phase with maintenance dose of fluconazole.

Pneumocystis jiroveci Pneumonia

Immune reconstitution inflammatory syndrome may present as worsening pulmonary symptoms and high fever in patients who had been improving on PCP therapy or in patients with recent successful treatment of PCP.

IRIS AND MALIGNANCY

Kaposi Sarcoma

- Kaposi sarcoma (KS) is a multifocal systemic tumor with origin in the endothelial cells which was first described by Moriz Kaposi in 1987.
- It is classified into four variants:
  1. Classic KS
  2. Endemic or African KS
  3. Iatrogenic KS
  4. AIDS-associated KS
- The AIDS-KS is the most prevalent form at present. Its first line therapy is ART.
- ***French et al*** defined IRIS-associated KS as:
  - An abrupt clinical worsening of a previously existing KS (paradoxical) or a new presentation of a previously unknown KS (unmasking) in temporal association with initiation or re-initiation of ART or change to a more active regimen
  - Concomitant reduction of at least 1 $\log_{10}$ in the HIV-1 RNA levels at the time of the IRIS event or with two of the following three minor criteria:
    1. Twofold increase in CD4+ T cell count after ART.
    2. Increase in the immune response (HHV-8 antibodies).
    3. Spontaneous resolution of disease without specific chemotherapy with continuation of ART.

Pathogenesis

- The synergy between the humoral and cellular immune response against HHV8 secondary to ART results in inflammation and tumorigenesis characterizing the clinical presentation of IRIS-KS.
- Most AIDS-KS patients have undetectable or very low HHV8-specific cytotoxic T lymphocytes.
- After the initiation of ART, CD8 T specific for HHV8 are detectable, and the recovery of this cell population is thought to be partly responsible for IRIS-KS.
- After ART therapy, there is an increase in the lymphocytes producing TNF-α, INF-γ and IL-1β which causes a reactivation of latent HHV8 and up-regulation of integrins like **MMP and VEGF.**
- The TAT protein encoded by the HIV virus binds to the Integrin receptors on the KS cells, thereby promoting mitogenic stimulus to VEGF.

Treatment

- Treatment with corticosteroids is not recommended because glucocorticoid treatment promotes HHV8 replication and tumor growth, worsening disease progression.
- The regimen with HAART and chemotherapy demonstrate an effective suppression of HHV8 viral replication.

Non-Hodgkin's Lymphoma

- Non-Hodgkin's lymphoma (NHL) have also been reported as a rare manifestation of immune reconstitution, however, literature is scant regarding lymphomas.

POINTS TO REMEMBER

- It is extremely important to be aware that ART-engendered immune recovery may result in pathological inflammation in a subset of patients.
- Vigilance needs to be especially high during the first several months of therapy when the incidence of IRIS peaks.
- However, cases continue to occur even after 1 or 2 years of therapy.
- More studies are needed to elucidate the pathogenesis and establish better treatment modalities.
- IRIS is a clinical entity requiring high level of suspicion, though not fatal, still requiring supportive care.
- Due to the lack of properly laid down diagnostic criteria, this entity is mostly underdiagnosed.

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4 Principles and Clinical Utility of Measuring Immunoglobulin Subclasses

Poorvi Kapoor

INTRODUCTION

- Immunoglobulin is a glycoprotein that is made in response to an antigen and can recognize and bind to the antigen that caused its production.
- They derive their name from the finding that they migrate to the region of globulins, when serum is placed in an electrical field.
- They are gamma globulins secreted from plasma cells.
- They constitute 25–30% of total serum proteins, and are present in serum, tissue fluids and mucosal surfaces.

BASIC STRUCTURE

- They are composed of two heavy and two light polypeptide chains, which are linked by disulphide bonds.
- These chains are subdivided into constant and variable regions, which contain an amino terminal in the variable region and carboxy terminal in the constant region.
- Light chains occur in two varieties, kappa and lambda and have a single variable (VL) and single constant domain (CL).
- Heavy chains, on the other hand, have a single variable domain (VH) and three constant domains (CH1, CH2, CH3) and the hinge region is seen between the CH1 and CH2 domains.
- On digestion with proteolytic enzymes (like papain), the peptide bonds in the hinge region are broken and produce three fragments.
- Two identical fragments, known as Fab, have antigen binding activity, and the other Fc fragment has effector functions and is responsible for complement fixation, opsonization and placental transfer.

Classification

They are classified into 5 types based on differences in the amino acid sequences of the constant portion of the heavy chains:

- IgG (gamma)
- IgA (alpha)
- IgM (mu)
- IgD (delta)
- IgE (epsilon).

Immunoglobulin G

- It is the most abundant class in the serum and constitutes 80% of total immunoglobulins.
- It contains less carbohydrates than other immunoglobulins and has a half-life of 23 days, which is the longest of all Ig subtypes.

Functions

- It crosses the placenta and provides immunity to the fetus and neonate at birth.
- It acts against bacteria and viruses by opsonization.
- Activates complement via the classical pathway.
- It has 4 subclasses which vary in the number of disulphide bonds and length of the hinge region: IgG1, IgG2, IgG3 and IgG4.
- IgG1, IgG3, IgG4 cross the placenta and protect the fetus.
- IgG1 and IgG3 bind Fc receptor on phagocytic cells and mediate opsonization.

Immunoglobulin A

- It is the second most common globulin and constitutes 10–15% of total immunoglobulins.
- It is present in milk, saliva, tears, mucosa of respiratory tract, digestive tract and genitourinary tract and is present as a monomer in serum, and a dimer in secretions.
- The J chain is associated with the dimeric form, has a half-life of 6–8 days and has two subclasses, IgA1 and IgA2
- The secretory piece is made in epithelial cells and is added to the IgA (made in plasma cells), as it passes into the secretions.
- This secretory piece helps IgA to be transported across mucosa and also protects it from degradation.

Functions

- It provides local immunity and provides an important line of defense against *S. typhi*, *V. cholerae*, *N. gonorrhoeaes* as well as influenza and polio virus.
- IgA secreted in breast milk protects the infant in the first few months of life.
- It activates complement via the alternate pathway and promotes phagocytosis and intracellular killing of organisms.

Immunoglobulin M

- It constitutes 5–10% of total serum proteins and is a polymer of five monomeric units (pentamer).
- It is held together by disulphide bonds and the J chain.
- IgM is mostly present in the intravascular compartment, has a half-life of 5 days and is the first immunoglobulin to be produced in primary response to an antigen.
- It is relatively short lived and therefore its demonstration in the serum indicates a recent infection.
- When detected in the serum of newborns, it indicates a congenital infection.

Functions

- It agglutinates bacteria and activates complements via the classical pathway.
- It is responsible for opsonization and is also responsible for immune-mediated hemolysis.

Immunoglobulin E

- Its structure is similar to IgG and it has a half-life of 2 days
- It is mostly present in extracellular location
- It is heat labile with a serum concentration of 0.3 μg/mL.

Functions

- It binds to the Fc receptor on the membranes of basophils and mast cells and mediates the release of histamine and other mediators of anaphylaxis
- It is responsible for anaphylactic ractions, hay fever, asthma and immediate hypersensitivity
- It also plays a role in immunity against helminths.

Immunoglobulin D

- Its structure is also similar to IgG and it constitutes 0.2% of total immunoglobulins
- It has a half-life of 3 days and is found in low levels in the serum
- Its role in the serum is uncertain, but it is primarily found on B cell surfaces where it functions as a receptor for antigens

Clinical Utility of Measuring Ig Subclasses

- The diagnosis of immunodeficiency is difficult, some of which include lack of screening, extensive use of antibiotics masking clinical presentations and lack of specific physical abnormalities associated with deficiencies.
- Immunodeficiency states should be suspected when there is:
  - ≥1 systemic bacterial infections (sepsis, meningitis, etc.)
  - ≥2 serious respiratory or documented bacterial infections like cellulitis, otitis media, pneumonia, lymphadenitis, etc. within 1 year
  - Unusual sites of infection like the brain or liver
  - Or unusual pathogens like *P. jiroveci*, *Aspergillus*, *S. marcescens*, *Nocardia* or *Burkholderia*
- The European Society of Immunodeficiency (ESID) has stated 10 warning signs of immunodeficiency.
  1. ≥8 new ear infections within 1 year
  2. ≥2 serious sinus infections within 1 year
  3. ≥2 months of antibiotic intake with little effect
  4. ≥2 pneumonias within 1 year
  5. Failure of an infant to gain weight or grow normally
  6. Recurrent deep skin or organ abscesses
  7. Persistent oral thrush or fungal infections of the skin
  8. Need for IV antibiotics to clear infections
  9. ≥2 deep seated infections
  10. A family history of primary immunodeficiency.
- Age of presentation varies depending on the cause of immunodeficiency:
  - Onset <6 months of age suggests a T cell defect
  - Between 6–12 months may help suggest a combined B and T cell defect
  - Presentation after 1 year of age suggests a defect in immunoglobulins

- Common defects can either have a known or unknown genetic basis
  - *Known genetic basis:*
    - Bruton agammaglobulinemia
    - Hyper-IgM syndrome
  - *Unknown genetic basis:*
    - Common variable immunodeficiency
    - Selective IgA deficiency
    - IgG subclass deficiency
    - Transient hypogammaglobulinemia of infancy.

Bruton's (X-Linked) Agammaglobulinemia

- It is due to mutation in Bruton tyrosine kinase and occurs in the later part of the first year of life.
- There are recurrent bacterial infections, usually with Pneumococci, Streptococci, Meningococci, *Pseudomonas* and *H. influenzae*.
- All the classes of immunoglobulins are grossly depleted and there is a marked decrease of B cells in circulation, with no formation of antibody even after injecting antigens.
- The tonsils and adenoids are atrophic but the thymus is normal.

Hyper-IgM Syndrome

- It can be X-linked which is associated with CD40 ligand defect (HIGM-1), with these cases associated with *P. carinii* and autoimmune disease.
- The autosomal recessive form is associated with mutation in gene cytidine deaminase (HIGM-2). There is lymphoid hyperplasia.
- Low IgG and IgA levels are seen with elevated IgM.
- There is hemolytic anemia with neutropenia and thrombocytopenia along with renal lesions.

Common Variable Immunodeficiency

- It occurs between 15–35 years of age.
- There is late onset hypogammaglobulinemia with recurrent pyogenic infection and an increased incidence of autoimmune disease.
- The total immunoglobulin levels are low with defective B cells in circulation.
- There is an increase in suppressor T cell with decreased helper T cell activity.

IgA Deficiency

- It can be inherited in an autosomal recessive or dominant pattern and occurs in 1 in 400 to 3000 individuals.
- There may be no presenting symptoms or they can present with sinopulmonary or gastrointestinal infections like giardiasis.
- It is characterized by serum IgA values < 10 mg/dL along with normal values of IgM and IgG.
- They may develop autoimmune diseases and can present with atopy or allergy to cow's milk.
- There is a risk of developing antibodies to IgA upon receiving blood products.

IgG Subclass Deficiency

- This deficiency is clinically very heterogeneous and co-exists with abnormal expression of > 1 subclass antibody, of which IgG2 or IgG3 deficiencies are the most common
- Clinically, they present with frequent bacterial infections of both upper and lower gastrointestinal tracts
- Since IgG1 is the most abundant subclass, a fall in the value of IgG1 causes total IgG to fall below normal, thereby causing hypogammaglobulinemia
- IgG4 levels are usually quite low in children, so a deficiency of this subclass presents later in life, usually after 10 years of age.

Transient Hypogammaglobulinemia of Infancy

- Maternally transmitted IgG gets catabolized by the 3rd or 4th month of life
- Symptoms occur due to slow maturation of the immune system which leads to a delay in immunoglobulin production
- Clinical presentation includes recurrent otitis media and respiratory tract infections
- Spontaneous recovery occurs between 18–30 months of age.

There are certain conditions which are associated with increase in immunoglobulin levels.

Conditions Associated with Increased IgE

Allergic Bronchopulmonary Aspergillosis

- An elevated level of serum IgE >416 IU/mL is one diagnostic criteria
- Levels of IgE are used to monitor the course of the disease and these levels fall with successful treatment with steroids and raise during disease exacerbation
- There is an association between disease activity and levels of Aspergillus antibodies

Asthma

- Atopic asthma is strongly related to increased levels of serum IgE
- A positive family history and skin test with the offending antigen induces an immediate wheal and flare response
- An anti-IgE monoclonal antibody, omalizumab, is an approved drug for use in asthmatics.

HyperIgE Syndrome

- It is an autosomal dominant disorder and is associated with a mutation of STAT3
- The autosomal recessive variant is associated with TYK2 and DOCK8 mutation
- These cases present with recurrent infections with *Staphylococcus aureus* and *Candida albicans*
- There are associated abnormalities like prominent forehead, hypertelorism, broad nasal bridge, facial asymmetry and hemihypertrophy
- IgE values are well above 2000 IU/mL and there is associated eosinophilia. They are also associated with an increased risk of lymphomas.

Conditions Associated with Increased IgM

Immunodeficiency Associated with HyperIgM

- It is an X-linked disorder associated with CD40 ligand defect (HIGM1) with an increased susceptibility to *P. carinii* and other autoimmune diseases.
- The autosomal recessive inheritance is associated with a mutation in cytidine deaminase (HIGM2) and is associated with lymphoid hyperplasia
- Elevated IgM levels are seen with low IgA and IgG levels.
- There can also be associated hemolytic anemia, neutropenia, thrombocytopenia and renal lesions.

Primary Investigations

- **Clinical history**
  - Recurrent and persistent infections
  - Infections due to unusual microbes
- **Physical examination**
  - Pale, lethargic, appearing chronically ill
  - On examination, there may be small or absent peripheral lymph nodes, tonsils or adenoids in infants and children
  - There may be petechiae and eczematous rashes over the skin
- **Complete blood count**
- **Radiological imaging**
  - X-rays show chronic interstitial infiltrates, absence of thymic shadow and bony abnormalities
  - CT scan may show pulmonary fibrosis and bronchiectasis, especially in CVID
- **Quantitative immunoglobulin assay**
  - Serum IgG, IgM and IgA levels
  - IgG subclass analysis.

Role of Immunoglobulins in Plasma Cell Disorder

- Plasma cell disorders are neoplastic proliferations of plasma cells with the production of immunoglobulins
- Various plasma cell disorders are as follows:
  - Multiple myeloma
  - Monoclonal gammopathy of undetermined significance
  - Asymptomatic multiple myeloma
  - Light chain multiple myeloma
  - Non-secretory myeloma
- Myeloma cells secrete IgG in 60% cases, less often IgA (20%), light chains (15%) and very rarely IgD, IgE and IgM
- It is important to evaluate the M component on electrophoresis for diagnosis
- Serum free light chain (SFLC) assay is useful for diagnosing, monitoring as well as for prognostication in these conditions
- This assay measures kappa and lambda ratio, with the normal ratio ranging between 0.26 to 1.65
- The diagnostic sensitivity of SFLC assay is highest for multiple myeloma (96.8%) and lowest for monoclonal gammopathy of undetermined significance (42%)

- Serum electrophoresis, nephelometry, radial immunodiffusion and immunofixation are the methods commonly used for SFLC assaying.

Nephelometry

- In this method, the light which is scattered from the antigen-antibody complex is measured
- **Principle:**
  - On the passage of an electromagnetic radiation (light), some amount of light is absorbed by the solution, and some is scattered or reflected
  - The scatter of this light is proportional to the concentration of insoluble particles present in the solution
  - When a sample containing an antigen is added to a cuvette containing the corresponding antisera, there is formation of an antigen-antibody complex
  - A laser diode then generates a beam of light, which is passed through this cuvette and the scatter is measured
  - A reference curve is generated from a standard containing known quantities of antigen
  - The scattered light can then be read from this curve
  - The intensity distribution of the scattered light depends on the relationship of the particle size of the generated complex to the wavelength of light used
- **Factors affecting nephelometry:**
  - Particle size and concentration (preferred for small particles in low concentration)
  - Distance of the light source
  - Wavelength of light used
- **Applications**
  - It is used primarily to measure the concentrations of immunoglobulins and serum proteins (IgG, IgA, Albumin, etc.)
  - It can also be used to monitor therapeutic drug levels in blood samples.

Radial Immunodiffusion

- It is a single diffusion technique used where the antibody is put into the gel and antigen is measured by the size of the precipitin ring formed when it diffuses out in all directions
- **Principle:** An antigen (in the sera of patients) is added to an antibody rich media and the antigen keeps reacting with the antibody till the zone of equivalence is reached. The area of ring is a measure of the concentration of the antigen present.

Serum Electrophoresis

- In this technique, the patient's serum is placed on a supporting medium made of cellulose or agar, which is then placed in an electrophoresis chamber and exposed to an electric current.
- The serum proteins get separated into 5 components: albumin, alpha 1 and alpha 2 globulin, beta and gamma globulins in the form of bands

Immunofixation

- It is used in serum or urine samples to identify the nature of M protein and is very sensitive.

- In this method, monospecific antibodies are used to identify specific types of immunoglobulin and light chains.
- The serum is placed on agarose gel in 5 different lanes and monoclonal antibody is then applied over the surface of the gel. The formed antigen–antibody complex is then visualized by staining.

Treatment

- Antimicrobial prophylaxis
- Immunization (all live vaccines avoided)
- Immunoglobulin therapy

POINTS TO REMEMBER

- Immunoglobulin is a glycoprotein that is made in response to an antigen and can recognize and bind to the antigen that caused its production.
- IgA deficiency is more common, followed closely by IgG.
- The ten warning signs of deficiency should be kept in mind and onset of these diseases usually occur after 1 year of age.
- Immunodeficiency diseases are difficult to diagnose, especially due to extensive use of antibiotics masking the classical presentation.
- These deficiencies require extensive clinical and laboratory tests to confirm the diagnosis.

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5 Microparticles

WHAT ARE MICROPARTICLES?

- First described in 1967 by Wolf as platelet membrane fragments in human plasma.
- Microparticles (MPs) are circulating phospholipid rich, submicron particles (0.05 –1.5 μm in size).
- They are released from the membranes of endothelial cells, platelets, leucocytes and erythrocytes.

Characteristics of Microparticles

- MPs are small particles.
- Their membranes consist mainly of lipids and proteins.
- They expose the anionic phospholipid phosphatidylserine.
- They also express membrane antigens that reflect their cellular origin and the cellular processes triggering their formation.
- Their shelf life is quite long (≈ 6 days).
- They are released from many blood cells.
- MPs bind to Annexin V, in the presence of Ca^{2++}.
- They carry CD markers, Tissue factor (TF), GP IIb-IIIa, etc.

Cellular Origin of Microparticles

- **Platelets** during activation of coagulation.
- **Endothelial cells** in auto-immune diseases, TTP and activation of coagulation.
- **Leucocytes** during the process of inflammation.
- **Erythrocytes** in cases of sickle cell anemia.

Generation of Microparticles

- Microparticles are released from the surface of cells following cell activation or apoptosis.
- The triggers include chemical stimuli, such as cytokines, thrombin and endotoxin, or physical stimuli, such as shear stress or hypoxia.
- Following cell activation, microparticle formation is dependent on a rise in the cytosolic calcium concentration with consequent activation of calpain and protein kinases and phosphatase inhibition.

- These changes result in cytoskeletal reorganization, membrane blebbing and the formation of microparticles.
- All these steps are shown in Fig. 5.1.

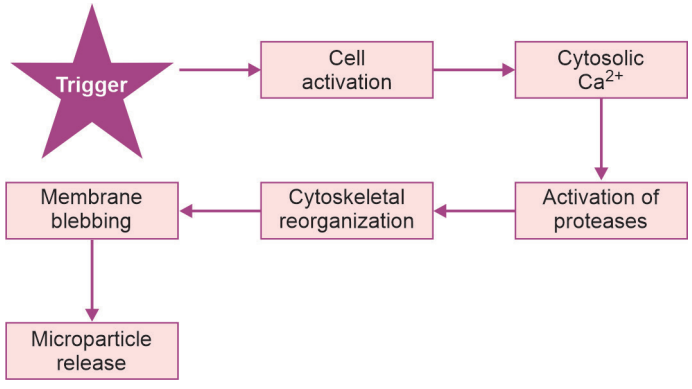

Fig. 5.1: Steps of generation of microparticles

Platelet Microparticles

- The platelet derived MPs are often seen at the site of blood vessel injury.
- These contribute to the process of hemostasis by providing a large surface for activation of the coagulation cascade leading to formation of the fibrin clot.
- MPs harbor active tissue factor (TF). Together they act synergistically as potent triggers of blood coagulation with TF-bearing MPs representing the so-called blood-borne TF.
- In contrast, pathologic MPs at high concentrations can predispose to thrombosis.
- Thus, they can be viewed as a major therapeutic target for arterial thrombosis, venous thrombosis and atherosclerosis.
- Apart from their procoagulant function MPs could also be involved in anticoagulant activity.
- It is due to the presence of tissue factor pathway inhibitor (TFPI) and antithrombin activity.

Endothelial Microparticles

- Microparticles have been shown to be released during apoptosis induced *in vitro* by growth factor deprivation or complement proteins.
- Both the cell of origin and the nature of the trigger influence the number and phenotype of the microparticles released and, consequently their pathophysiological effects.
- For example, endothelial microparticles (EMPs) released following apoptotic stimuli have higher levels of surface Annexin V binding to phosphatidylserine, and of constitutive endothelial cell markers such as CD31 (Platelet Endothelial Cell Adhesion Molecule, PECAM).
- EMPs induced by activation with TNF-α expressed higher levels of inducible antigens, such as CD62E (E-selectin), which were also increased on the parent endothelial cells.
- Total EMPs levels can be used as a surrogate marker of endothelial disturbance.
- The phenotypic profile of EMPs can help to discriminate between endothelial activation and apoptosis.

- For example, ratio of CD62+ (E-selectin) EMPs to CD31+ (PECAM) EMPs has been shown *in vitro* to be high in activation, low in apoptosis.
- Previously MPs were considered inert cell debris but now there is a growing evidence from *in vitro* and some *in vivo* studies that MPs may exercise a number of pathophysiologic functions.

MPs Functions

- Intercellular transfer of membrane proteins and lipids.
- Prothrombotic effects
- Proinflammatory effects
- Impairment of endothelial function
- Modulation of vascular tone
- Induction of angiogenesis and enhancement of cancer cells metastasis.

Role in Endothelial Function

- Endothelial microparticles have been shown to reflect endothelial activity, being released following activating or injurious external stimuli, and to themselves induce changes in endothelial function.
- Endothelial dysfunction is a common feature of many vascular disorders including atherosclerosis, diabetes, antiphospholipid syndrome, TTP and sickle cell disease.
- Endothelium-derived nitric oxide (NO) is the major mediator of acetylcholine-induced vasorelaxation of rat aorta *in vitro*.
- Exposure of rat aorta to EMPs obtained from cultured endothelial cells, resulted in impaired acetylcholine induced relaxation and reduced NO production.
- The same effect was also observed when using circulating microparticles obtained from patients following myocardial infarction (MI).
- Circulating EMPs may also contribute to the vascular changes seen in end stage renal disease through inhibition of the endothelial NO pathway.

Cellular Interactions

- Microparticles bear antigens of their cell of origin and can transfer these surface molecules to other cell types.
- The binding of microparticle surface antigens to their specific counter-receptor may induce intracellular signaling pathways and may alter the biological activity of the recipient cells.
- They are the consequence of the disease but can also be the cause of further clinical complications, by stimulating the blood procoagulant pathways.

Association of MPs with Diseases

- They have been reported to be associated with following diseases:
  - Hypertension
  - Diabetes
  - Myocardial infarction
  - Cancer
  - Acute coronary syndrome
  - Lupus anticoagulant

- Sepsis
- Pre-eclampsia.

- There is insufficient evidence to demonstrate that this association is causal.
- Definitely, elevated blood MPs in several disease processes result from inflammation, cellular activation and apoptosis.

Microparticles and Blood Transfusion

- In stored human blood, MPs are derived from platelets, leukocytes, and erythrocytes.
- Erythrocyte and platelet derived MPs have been implicated as mediators for:
  - Transfusion-related acute lung injury (TRALI)
  - Febrile nonhemolytic transfusion reactions
  - Thrombocytopenia
  - Urticaria
  - Thrombosis.

MICROPARTICLES: AS DIAGNOSTIC MARKERS

- Elevated in myocardial infarction (two to ten times)
- Increased in stroke.
- Increased levels predict vascular complications in diabetes
- Elevated levels have been also observed in certain cancers, for example, carcinoma breast.
- MPs levels correlate well with severity of hypertension.
- MPs are useful in follow-up and therapeutic monitoring of patients with myocardial infarction.
- Up to ten times elevation has been reported in hemophilia.
- MPs derived from PLTs, RBCs, WBCs, and endothelial cells have been found to be elevated in sickle cell disease and PNH.

Microparticle Measurement

Different methodologies available for MPs measurement are as follows:

- Immunoassay by ELISA capture
- Flow cytometry.

Immunoassay by ELISA Capture

Sample Collection

- Blood collected in 0.109 M citrate anticoagulant (9 vol blood in 1 vol trisodium citrate), through a clean venipuncture, avoiding blood activation.
- Plasma is prepared by blood centrifugation at 1,500 g for 15 min at room temperature (18–25°C), then plasma decantation (PPP).
- Centrifugation of plasma (PPP) is done for 2 min at 13,000 g at room temperature (18–25°C).
- It is possible to keep the specimen frozen (various months/years).

Assay Principle

- Micro ELISA plate is coated with streptavidin and Annexin V then washed.
- Diluted tested plasma is introduced in a microwell, in presence of calcium.

- When present, microparticles (MPs) exposing anionic phospholipids bind to Annexin V.
- Following a washing step, first FXa and Va are introduced, with calcium, then prothrombin.
- Generated thrombin is measured using a specific chromogenic substrate, and absorbance is measured at 405 nm .

Results

- MPs in normal individuals are < 10 nmol/L.
- In various pathologies the concentration is 2 to 10 times those of normal plasma.

Advantages

- ELISA is available at most research institutions.
- It is less costly than flowcytometry.
- Not restricted by size as it measures all MPs including those that are < 0.4 or 0.1 μ).

Disadvantages

- Analysis is done in batches and thus takes longer time.
- No size determination is possible.

Flow Cytometry

- Flow cytometry relies on the antigenic composition of MPs and allows them to be enumerated according to their cellular origin.
- Only large microparticles (> 0.4 μ) are measured as the size and content are important parameters.
- The microparticle source, surface antigen expression and proportion in circulation are shown in Table 5.1.

Table 5.1: Microparticle source, surface antigen expression and proportion in circulation

Cellular source of MPs	*Marker*	*Proportion in circulation*
Platelets	CD61 (GPIIIa) CD63 CD62p (P-selectin) CD41	80–90%
Leucocytes	CD45	< 5%
Erythrocytes	Glycophorin A	5–10%
T helper cells	CD4	< 1%
T cytotoxic cells	CD8	< 1%
B cells	CD20	< 1%
Monocytes/macrophages	CD14	< 5%
Endothelial cells	CD62e (E-selectin)	< 5%

Advantages

1. Rapid turnaround time.
2. Both fresh and frozen specimens may be used.

3. The expression of two or more antigens on the MPs may be simultaneously demonstrated.
4. Easy method for quantification using commercial beads.

Disadvantages

1. The detection of particles less than 0.3 μm is difficult by flow cytometry.
2. Different machines have different sensitivities.
3. Centrifugation speeds for sample processing are variable and not standardized.

Other rarely used methods

- Functional assays
- Nanoparticle analysis
- Atomic force microscopy

MICROPARTICLES: GOOD OR BAD

- MPs are noxious elements, supporting proinflammatory, procoagulant potential and inhibiting vascular repair.
- Increased levels of MPs of endothelial origin in various pathologies, such as atherothrombosis, vasculitis, and sepsis have been seen.
- However, these EMPs have a beneficial effect on endothelial integrity, such as stimulation of vascular repair, control of cell death mechanisms and cytoprotective activities.
- Neutrophil-derived MPs release potent anti-inflammatory effectors (such as annexin A1) at the onset of an inflammatory response.

Recent Developments

- Some currently used therapies are known to affect microparticle generation.
- For example, abciximab, a glycoprotein IIb–IIIa receptor antagonist.
- It is currently used as an antiplatelet drug in prevention of ischemic complications after percutaneous coronary intervention.
- Also, it almost completely blocks platelet vesiculation *in vitro*.
- Treatment of patients suffering from transient ischemic attacks with calcium channel blockers has been shown to decrease microparticle generation.
- A randomized, placebo-controlled, double blind trial showed that treatment of patients with congestive heart failure with vitamin C decreased the number of circulating microparticles.
- Cilostazol (a selective cyclic AMP phosphodiesterase inhibitor and antiplatelet agent) and ticlopidine (an ADP antagonist) decrease platelet-derived MPs.
- Eposartan an antihypertensive agent, lowered or normalized numbers of monocyte and platelet derived MPs.

POINTS TO REMEMBER

- Cellular MPs are submicrometric fragments resulting from the remodeling of plasma membrane in response to numerous conditions, including activation and apoptosis.
- MPs may prove useful as circulating biomarkers of endothelial dysfunction and

prothrombotic state, both in disease and to monitor the effects of treatment, such as statins in vascular diseases.

- The understanding of pathophysiological relevance of blood MPs is still in its infancy.
- Future studies are needed to provide additional evidence of the role of MPs in various disease states.
- This may lead to development of additional interventions and strategies for the prevention and treatment of various disorders.

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Recent Concepts in Tumor Immunology

6

Tanisha Singla

INTRODUCTION

Immune Surveillance

- Immune surveillance is a theory that the immune system patrols the body not only to recognize and destroy invading pathogens but also host cells that become cancerous.
- When a tumor escapes immune surveillance and grows too large for the immune system to kill, cancer occurs.

Evidence for Immune Reactivity to Tumors

- Tumors that have lymphocytic infiltration have a better prognosis than those that do not.
- Many lymph nodes draining tumor sites show reactive changes.
- Certain tumors regress spontaneously (e.g. melanoma, neuroblastoma).
- There is an increased incidence of primary and secondary malignancies in immunodeficient patients.
- The young and the very old have an increased occurrence of tumors.
- These members of the population often have an immune system that is less effective.

Tumor Antigenicity

- Next-generation sequencing has revealed that many somatic mutations occur in the DNA of cancer cells that were not known previously.
- These mutations occur in the coding regions of genes and are transcribed into RNA, which is then translated into proteins.
- Fragments of these mutated proteins **appear as peptides displayed by HLA molecules** on the surface of tumor cells, where they can be recognized as **"foreign" peptides** by T cells.
- These tumor antigens are useful tumor markers in identifying tumor cells and are potential candidates for use in cancer therapy.

IMMUNOGENICITY OF TUMORS

Two types of tumor antigens:

- **Tumor associated transplantation antigens (TAA):** These are found on tumor cells and on normal cells during fetal life (oncofetal antigens), after birth in selected organs at low concentration.
- **Tumor specific transplantation antigens (TSTA):** Present only on tumor cells, but not on normal cells
- Most chemically- or physically-induced tumors or those produced as a result of a virus, have **neoantigens**.
- Spontaneously occurring tumors are often weakly immunogenic or non-immunogenic.
- Antigenic changes observed in malignant cells include:
  - Reappearance of fetal antigens (oncofetal antigens)
  - Expression of unique antigens not expressed by normal cells.

Oncofetal Antigens

- Oncofetal antigens may appear due to de-repression of genes that were only expressed early in life.
- Two major oncofetal antigens are **alpha-fetoprotein** (**AFP)** and **carcinoembryonic antigen** (**CEA**).
- Others include—pancreatic oncofetal antigen, fetal sulfoglycoprotein.
- Studies have shown that expression of oncofetal antigens in adults is not limited to tumors.
- These proteins are increased in tissues and in the circulation in various **inflammatory conditions**, and they are also found in **normal tissues**.
- There is no evidence that oncofetal antigens are important inducers or targets of antitumor immunity.
- However, they can serve as markers that aid in tumor diagnosis and clinical management

Tumor Antigens Produced by Oncogenic Viruses

- Viruses produce proteins that are recognized as foreign by the immune system.
- The most potent of these antigens are proteins produced by latent DNA viruses.
- Example: Human papillomavirus (HPV) E6 and E7 proteins in cervical cancer.

Products of Mutated Genes

- Neoplastic transformation occurs due to genetic alterations in proto-oncogenes and tumor suppressor genes.
- These mutated genes encode variant proteins which are recognized by the immune system as nonself.
- Example: Mutated oncoproteins like RAS and BCR/ABL fusion proteins.
- Mutated tumor suppressor gene products like mutated p53 protein.

Altered Cell Surface Glycolipids and Glycoproteins

- Tumors express higher than normal levels and/or abnormal forms of surface glycoproteins and glycolipids, which may be diagnostic markers and targets for therapy, e.g. CA-125 and CA-19–9 expressed on ovarian carcinomas.

Cell Type-specific Differentiation Antigens

- Tumors express molecules that are normally present on the cells of origin. These antigens are called ***differentiation antigens*** because they are specific for particular lineages.
- These are normal self-antigens therefore they do not induce immune responses.
- They are potential targets for immunotherapy and for identifying the tissue of origin of tumors.
- Antibodies against CD20 (Rituximab) can be used in the treatment of B cell leukemia and lymphoma.

IMMUNITY AGAINST TUMORS

- Evidence for immunity against malignancy comes mostly from experimental models.
- In experimental studies, animals can be immunized by administering inactivated tumor cells or by removal of a primary tumor.
- Immunity can be transferred from an animal, in which a tumor has regressed, to a naive animal by injection of lymphocytes (T cells).

MECHANISMS OF IMMUNE SURVEILLANCE

- All components take part- NK cells, cytotoxic T lymphocytes (CTL) macrophages.
- Out of these macrophages play predominant role.

Natural Killer Cells (NK) and Antibody-Dependent Cellular Cytotoxicity

- NK cells are effector cells in an innate immune response.
- They play a dynamic role in the nonspecific killing of tumor cells or virus-infected cells
- NK cells can be moved to kill target cells that do not express MHC class I molecules.
- Two cell surface molecules, CD16 and CD56, are commonly used to identify NK cells.
- CD16 is an Fc receptor for IgG, and it confers on NK cells the ability to lyse IgG-coated target cells.
- This phenomenon is known as **antibody-dependent cell-mediated cytotoxicity (ADCC).** (The function of CD56 is not known)
- The functional activity of NK cells is regulated by a balance between signals from activating and inhibitory receptors.
- There are many types of activating receptors, of which the **NKG2D family** is the best characterized.
- The NKG2D receptors recognize surface molecules that are induced by various kinds of stress, such as infection and DNA damage.
- NK cell inhibitory receptors recognize self Class I MHC molecules, which are expressed on all healthy cells. The inhibitory receptors prevent NK cells from killing normal cells.
- Virus infection or neoplastic transformation enhances expression of ligands for activating receptors and reduces the expression of class I MHC molecules.
- As a result, the balance is tilted toward activation, and the infected or tumor cell is killed.

T Lymphocytes and Antitumor Immunity

- Effector mechanisms of tumor cell killing by cytotoxic T cells (CTLs) are as follows:
  - They can directly facilitate the lysis of tumor cells by identifying distinctive antigens presented by tumor cells.
  - They can kill tumor cells by signaling the induction of apoptosis in the target cells and by the secretion of perforins and granzymes.

B Lymphocytes and Antibodies

- The production of antitumor cell antibodies does not play a dominant role in host antitumor immune responses.
- However, monoclonal antibodies that are responsive to tumor-associated antigens show usefulness in antitumor therapy and for tumor detection.

Macrophages, Monocytes, and Dendritic Cells

- Monocyte/macrophages play important roles in immune responses.
- Macrophages, which form an important part of innate immune responses, also play a key role in the generation of adaptive immune responses, because they can act as active ***antigen-presenting cells***.
- Helper (CD4) T and CD8 T lymphocytes can be activated by antigen-presenting macrophages that show processed antigen joint with self MHC molecules.
- Antigen presenting cells also deliver key costimulatory signals that are important for T-lymphocyte activation.
- Macrophages can also ingest and kill microorganisms, and can act as cytotoxic, antitumor killer cells.
- Macrophages and monocytes produce various **cytokines and chemokines**, which are involved in both innate and adaptive immune reactions.
- These chemokines and cytokines have straight biologic effects on tumor cells - both as growth-inducing and growth-inhibiting factors.
- **Mature dendritic cells** are very effective antigen-presenting cells for T cells.
- They express very high levels of MHC class I and class II molecules, along with high levels of cell surface lymphocyte stimulatory molecules and adhesion molecules.
- They also secrete chemotactic factors (chemokines) that recruit T cells.

FAILURE OF IMMUNE SURVEILLANCE

- Cancers represent a rare failure of a system that has been eliminating transformed cells throughout life.
- If a cell succeeds in starting down the path leading to uncontrolled mitosis, it may acquire somatic mutations that protect it from attack by the host's immune system **(cancer immunoediting).**
- Examples:
  - Reduced expression of tumor antigens.
  - Reduced expression of class II MHC molecules needed to display tumor antigens to $CD4^+$ Th cells.
  - Reduced expression of class I MHC molecules needed to display tumor antigens to $CD8^+$ cytotoxic T lymphocytes (CTLs).
  - Reduced efficiency of loading antigenic peptides into MHC molecules.

- Secretion of immunosuppressive cytokines (TGF-β, IL 10).
- Recruitment of immunosuppressive T regulatory cells.

Can the immune system be manipulated to target cancer?

- The rapidly advancing field of cancer immunology has produced several new methods of treating cancer, called immunotherapies, that increase the strength of immune responses against tumors.
- Immunotherapies either stimulate the activities of specific components of the immune system or counteract signals produced by cancer cells that suppress immune responses.

IMMUNOTHERAPY

Immune Checkpoint Modulators

- Immune checkpoint modulators are proteins that normally keep immune responses in check by preventing overly intense responses that might damage normal cells as well as abnormal cells.
- Some tumors can commandeer these proteins and use them to suppress immune responses.
- Blocking the activity of immune checkpoint proteins releases the **"brakes"** on the immune system, increasing its ability to destroy cancer cells.
- The first drug to receive FDA approval, I**pilimumab (Yervoy®)**, for the treatment of melanoma, blocks the activity of a checkpoint protein known as CTLA4, which is expressed on the surface of cytotoxic T lymphocytes.
- CTLA4 acts as a "switch" to inactivate these T cells, thereby reducing the strength of immune responses.
- **Mechanism:** Cancer cells produce antigens which are recognized by dendritic cells and are presented to cytotoxic T cells which are then destroyed.
- However along with the antigens, the dendritic cells also present inhibitory signals that binds to CTLA-4 receptor present on cytotoxic T cell which turns **off** the cytotoxic mechanism leading to survival of the tumor cells.
- Ipilimumab binds to CTLA-4 receptor, thus blocking the inhibitory signal.
- Two other FDA-approved checkpoint inhibitors-nivolumab (Opdivo® and pembrolizumab (Keytruda®), work in a similar way, but they target a different checkpoint protein-**PD-1 (programmed cell death protein 1)**.
- Nivolumab is approved to treat patients with malignant melanoma and lung cancer.
- Pembrolizumab is approved to treat patients with malignant melanoma.

Adoptive Cell Transfer: CARs and TILs

- Another form of immunotherapy that is being actively studied is called adoptive cell transfer (ACT).
- In several small clinical trials testing ACT, some patients with very advanced cancers primarily **melanoma and blood cancers like leukemia and lymphoma** have had their disease completely eradicated.
- In ACT the patient's autologous T cells are expanded and manipulated *ex vivo* which are then reinfused into the patient to exert anti-tumor response.

CAR T cell Therapy

- In this form of ACT, patients' T cells are collected from the blood.
- These T cells are then genetically modified in the laboratory to express a synthetic receptor on their surface known as a **chimeric antigen receptor (CAR)**.
- CARs are of two types—**extracellular** single-chain variable fragments (ScFv) for antigen recognition and **intracellular** signaling domains for activating T cells.
- In CAR-T cells, the extracellular domain ScFv is responsible for assigning the specificity of CTLs to the malignant cells.
- CAR intracellular signaling domains provide the necessary signals for T cell activation.
- After the immune cells are engineered to express a CAR, they are then grown in the laboratory until there are hundreds of millions of them.
- The entire batch of CAR T cells is infused into the patient in a single dose.
- Due to the specificity to bind to the cancer cells - the modified T cells attack these cells.
- In 2017, **FDA approved the first CAR T cell therapy, tisagenlecleucel (Kymriah™)** for children and adolescents with acute lymphoblastic leukemia.

Tumor Infiltrating Lymphocyte (TIL) Therapy

- In this form of ACT-immune cells are collected from a patient's tumor-called tumor-infiltrating lymphocytes (TILs)
- TILs are immune cells that have naturally entered a tumor and their presence is thought to indicate that the immune system is trying to attack the cancer.
- Unlike CARs they do not undergo any further modifications or engineering.
- As with CARs and TCRs, large populations of these TILs are grown in the laboratory.
- The expanded TILs are then activated by treatment with cytokines.
- Then the activated cells are infused into the patient in a single dose.
- The idea behind this approach is that the TILs have already shown the ability to target tumor cells, but there may not have been enough of these immune cells around the tumor to eradicate it.
- Introducing massive amounts of activated TILs can help to overcome these barriers, leading to tumor destruction.

THERAPEUTIC ANTIBODIES

- Therapeutic antibodies are antibodies made in the laboratory that are designed to cause the destruction of cancer cells.

Monoclonal Antibodies in Immunotherapy (Magic Bullet)

- Monoclonal anti-tumor antibodies are used in different forms for the treatment of cancer.
- They are also used as vehicles to target anticancer drugs and toxins.
- Specific antibodies are also used in the diagnosis of metastatic lesions, which are not detectable by conventional radiological means.

IMMUNOCONJUGATES

- Tumor-targeted immunoconjugates consist of an **antibody and an effector moiety** which are bonded together.

- If the effector moiety is a cytotoxic drug, it is called **antibody–drug conjugate (ADC).**
- If the effector moiety is a protein toxin, it is called an **immunotoxin.**
- If the effector moiety is a radionuclide (toxic radioactive isotope), it is called a **radioimmunoconjugate.**
- Antibody drug conjugates consist of a cytotoxic drug conjugated to an antibody for targeting a specific antigen.
- The antibody binds to the specific antigen and is internalized by receptor-mediated endocytosis.
- Free drug is released in the intracellular compartment by lysozomal enzyme-mediated degradation of the drug–antibody conjugate whereby the drug restores its cell-killing potential
- USFDA has approved several ADCs for the treatment of patients with cancer, including:
  - Ado-trastuzumab emtansine **(**Kadcyla®) **for the treatment of breast cancer**
  - Brentuximab vedotin (Adcetris®) **for Hodgkin lymphoma**
  - Ibritumomab tiuxetan (Zevalin®) **for non-Hodgkin B cell lymphoma.**

Complement Binding Antibodies

- Monoclonal antibodies—Herceptin or Rituximab, cause selective cellular toxicity by binding to specific target antigen followed by cell lysis either by **antibody-dependent cellular cytotoxicity** or by complement activation and **complement-dependent cytotoxicity**.
- Rituximab (Rituxan®) targets CD20 which is expressed on surface of B cells.
- Used in the treatment of B cell lymphoma and leukemia.

Immunotoxins

- Immunotoxins are composed of a protein toxin attached to an antibody.
- Toxins are mainly cytotoxic enzymes. The antibody recognizes and binds to the specific tumor antigen and gets internalized.
- After the conjugate is internalized, the attached toxin begins its cytotoxic function and causes cell death.
- Example: Denileukin diftitox (**ONTAK®**) is approved for the treatment of cutaneous T cell lymphoma, contains a toxin produced by the bacterium *Corynebacterium diphtheriae*.

Antibody-Directed Enzyme Prodrug Therapy

- This includes the activation of a weakly toxic prodrug by an enzyme attached to the antibody at the tumor site to an active toxic drug.
- Results in humans have not been as positive as in experimental animals, but efforts continue to develop this approach into an effective therapeutic approach.

Cancer Stem Cells

- The existence of a small population of cancer cells called cancer stem cells which differentiate to form cancer cells may require targeting through **stem cell specific antigen** for specific eradication of these subtypes of cancer cells.

- Relapse of tumor cells after chemotherapy is associated with a small population of stem cells left behind unaffected by chemotherapy.
- These cells differentiate later to become the cancer cells which are resistant to previous therapy.
- Since stem cells are not rapidly dividing cells, conventional chemotherapy targeting rapidly dividing cells remains ineffective against cancer stem cells.
- Hence for a complete eradication of cancer, it is important to eradicate cancer cells along with cancer stem cells.
- This requires the identification of specific targets or exclusive pathways non-existent in normal cells but prevalent in cancer stem cells.
- This is still a developing concept and more research is going on.

Nonspecific Biological Products

- A variety of immuno-potentiating agents (**biological response modifiers**) are used to enhance anti-tumor immunity.
- They include:
  - bacterial products
  - synthetic chemicals
  - cytokines.
- Most of these agents exert their effects by activating macrophages and natural killer (NK) cells, eliciting cytokines or enhancing T cell functions.

POINTS TO REMEMBER

- Many mechanisms of the immune system can have active antitumor responses, including direct cytotoxicity directed against tumor cells along with the production of immune-enhancing cytokines.
- The mis-regulated expression of various genes, including overexpression of oncogenes, loss of anti-oncogenes and overproduction of growth factor and cytokines may be responsible in the development and progression of some cancers.
- Alteration of the environment and antitumor immune responses may also play a role in the development and growth of malignancies.
- Understanding the exact strategies that are involved in the development and growth of cancer will provide chances for the lucid design of effective antitumor immunotherapy.
- Several forms of experimental immunotherapy for cancers have been studied, including biologic response modifiers and cytokines.
- In recent years, great advances have been made in the development of suitable forms of monoclonal antibodies directed to cell-surface molecules expressed on tumor cells.
- Immunologic therapy of several types of cancer currently remains experimental.
- These therapies can expand what can be achieved with ideal chemotherapy and therefore could deliver an incremental development in patient management over current standards.

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7 Connexins and their Role in Tumour Biology

Harsh Batra

INTRODUCTION

- Cells in a tissue are connected to each other through intercellular junctions which can be of five types:
  i. Tight junctions
  ii. Desmosomes
  iii. Hemidesmosomes
  iv. Adherens junctions
  v. Gap junctions
- Existing in nearly every mammalian cell, ***Connexins***, or gap junction proteins are family of structurally related transmembrane proteins that assemble to form vertebrate gap junctions.
- Gap junctions form a few to hundred tightly packed channels that allow direct exchange of small molecules between adjoining cells.
- The diversity of function is attributed to the subset of connexins that are expressed in any one cell type.

NOMENCLATURE

- Connexins are abbreviated as either Cx or CX.
- They are named according to their molecular weights, e.g. Cx26 is the connexin protein of 26 kDa.
- However, this may lead to confusion when connexins from different species are compared, e.g. human Cx36 is homologous to zebrafish Cx35.
- Another system of nomenclature based on sequence similarity and length of the cytoplasmic domain.
- It designates connexins as GJ (for gap junction) α, β, and γ (subgroups).
- They are serially numbered in the order of their discovery within each subgroup. For example: CX43, was the first connexin discovered in the α subgroup.
- It is, therefore, designated as Gjα1.
- Cx32 was the first in the β subgroup: Gjβ1.
- A Gap Junction Conference (2007) was held in Elsinore, Denmark.
- It was decided to use the GJ nomenclature system for the genes that encode connexins.

- The participants wished to retain the connexin nomenclature for the encoded proteins using the weight of the human protein.

GENERAL PRINCIPLES ASSOCIATED WITH CONNEXINS

- Many tissues and cell types express two or more members of the connexin family.
- For example, keratinocytes express at least Cx26 (connexin26), Cx30, Cx30.3, Cx31, Cx31.1 and Cx43.
- Co-expression of multiple connexin family members within the same cell type allows for possible compensatory mechanisms to overcome the loss or mutation of one connexin family member.
- This principle has been demonstrated effectively in connexin-gene-ablation studies.
- It was observed that the prevalence of disease or the incidence of abnormal development is far less than might be predicted if no compensatory mechanisms existed between co-expressed connexin family members.
- In a study by Kelsell et al. it was shown that humans suffering from loss-of-function mutations in Cx26 are deaf.
- But no accompanying liver disease was reported since Cx26 and Cx32 are co-expressed in hepatocytes.
- Though two or more connexins may be co-expressed in the same cell, the resulting channels formed cannot always compensate for the loss or mutation of a connexin family member.
- For example, in skin, a subset of loss-of-function Cx26 mutations that result in deafness also manifest as skin disease.
- This is despite the fact that the epidermis is rich, with presence of multiple members of the connexin family.
- ***The most ubiquitously expressed connexin is Cx43.***
- Cx43 is endogenously expressed in at least 35 distinct tissues encompassing over 35 cell types.
- These include cardiac myocytes, keratinocytes, astrocytes, endothelial cells and smooth-muscle cells.

MOLECULAR STRUCTURE OF CONNEXINS

- Connexins are polytopic integral membrane proteins where the polypeptide backbone threads through the membrane four times (transmembrane).
- This yields:
  1. Two extracellular loops (EL-1 and EL-2).
  2. A cytoplasmic loop (CL) with both the N-terminus (AT) and the C-terminus (CT) exposed to the cytoplasm.
- The protein is made up of nine domains as follows:
  - N-terminus
  - Two extracellular loops (EL)—stabilized by intramolecular disulfide bridges
  - Four transmembrane domains
  - Cytoplasmic loop domain
  - C-terminus domain.
- Amongst these nine domains, sequence conservation is most evident within the N-terminus, two EL domains and four transmembrane domains.

- Conversely, the cytoplasmic loop and the C-terminus show high diversity in terms of size and post-translational modification.
- Connexins oligomerize into characteristic hexamers also known as '***connexons***' or 'hemichannels'.
- This helps in gap junction channel formation where two connexons of adjacent plasma membranes appose and dock against each other.
- Individual gap-junction channels are arranged in hexagonal arrays also called ***gap-junction plaques.***
- Diameter of channel is approximately 2 nm.
- Possible channel subtypes increase exponentially when two compatible connexins are co-expressed in the same cell and are capable of assembling both in homomeric and heteromeric fashion.
- For example, heteromeric Cx26/Cx32 connexons exist in the liver; Cx46/Cx50 connexons in the lens; Cx26/Cx30 connexions in the cochlea.
- Thus there can be homotypic, heterotypic and combined heterotypic/heteromeric arrangements.
- These permutation and combinations of connexins help in the diversity of functions in a particular cell type.
- Channels formed of heteromeric connexons have different properties from those of homomeric channels.
- Properties of heteromeric channels are manipulated by regulating the ratio of constituent connexins.
- This allows regulation of the permeability and conductance of gap junctions.

Biosynthesis

- Having a short half-life of only a few hours, connexins are regularly biosynthesized and degraded.
- They have a short half-life in order to respond to physiological requirements to either up- or down-regulate the extent of gap-junction coupling.
- This response mechanism is best exemplified in the myometrium, where it is proposed that steroid hormones promote the dramatic 5-fold increase in total gap junctions just prior to labor onset.
- Reciprocally, they are rapidly cleared following labor re-establishing a steady-state level in the uterus.
- Connexins are synthesized on ER-bound ribosomes and inserted into the ER cotranslationally followed by folding of the connexin protein while in the ER.
- Connexin oligomerization occurs throughout the transport of the connexons within the ER till the trans-Golgi network, where oligomerization is completed.
- Connexon hemichannels are packaged into vesicles (between the ER and the trans-Golgi network) and delivered to the membrane which can be microtubule dependent or independent.
- Most get inserted and interact via their extracellular loops to form intercellular channels.
- The individual channels aggregate in the membrane to form plaques or gap junctions.
- Some connexon hemichannels may remain uncoupled playing a role in cell life cycle.

Post-translational Modification

- Connexins may undergo various types of post-translational modifications, including phosphorylation, hydroxylation, acetylation, disulfide binding, nitrosylation, and palmitoylation.
- The most studied is phosphorylation.
- It is essential for proper control of formation and modulation of function of these channels.
- Phosphorylation by different kinases such as Src, PKC, and MAPKs is required for and affects connexin/connexon trafficking, assembly and disassembly, degradation, and gating (rapid opening and closing) of gap-junction channels.
- Effect of phosphorylation on channel gating is very specific.
- For example, phosphorylation of a connexin isoform such as Cx43 on different residues by the same kinase may lead to opposite effects with respect to enhancing or inhibiting gap junction function.
- Other connexin functions including control of growth and proliferation are also affected.

Half-life and Degradation

- Connexin proteins have a very short half-life of a few hours.
- Synthesis and delivery to the membrane is coupled to simultaneous gap-junction internalization and degradation.
- Newly delivered connexions localize to the periphery of the existing gap junctional plaques and "old" connexons to be degraded are present at the center of the plaque.
- Following internalization, these complexes undergo preliminary degradation and finally disassembly of gap junctions and connexions into individual connexions.
- Connexin proteins undergo complete degradation through proteasomes or lysosomes.

Connexin Interactions

- These proteins do not act in isolation in gap-junction complexes.
- They interact with many associated proteins that play essential roles in regulating the assembly, function, and life-span of connexins.
- The main interacting partners include cytoskeletal elements, junctional proteins and enzymes.

Interaction with Cytoskeleton

- Cytoskeleton elements including *Microtubules*, *Actin*, *Spectrin* and *Debrin* help in transporting connexins to the membrane and for their rapid turnover and replenishment.
- Cx43 directly binds to α- and β-tubulin.
- Microtubules at the cell-periphery co-localize with Cx43-based gap junctions.
- Interaction with microtubules is essential in allowing directed transport of newly synthesized connexion hemichannels to the plasma membrane.
- Microtubules also direct newly synthesized connexions to sites of pre-existing adherens junctions.
- Interactions of connexins with the actin cytoskeleton helps to stabilize gap junctions at the plasma membrane.

- Debrin (developmentally regulated brain protein) is a novel connexin-interacting cytoskeletal protein.
- It is an actin binding protein that mediates cellular polarity and formation of stabilized plasma membrane domains.
- It interacts with the carboxy-terminal end of Cx43 and stabilizes Cx43 gap junctions at the membrane.

Interaction with Junctional Proteins

- Interaction of gap junction proteins with proteins of the adherens and tight junctions leads to formation of large protein intercellular complexes containing multiple junctional molecules.
- These interactions form a regulatory backbone for various stages of the connexin life cycle—membrane insertion, localization, and plaque formation.
- For example, Zonula Occludens-1 (ZO-1) is a tight junction membrane-associated guanylate kinase which is involved in the organization and trafficking of gap junctions.
- ZO-1 has also been shown to regulate Cx43 mediated gap junctional communication in osteoblastic cells by altering its membrane localization.
- The C-terminal of Cx43 and Cx45 interact with the second PDZ domain of ZO-1, thereby playing a role in recruitment of regulatory protein to gap junctions.
- Furthermore, connexions have also been shown to have important interactions with occludin, claudin and cadherin.

Interaction with Enzymes

- Tyrosinases, serine/threonine kinases and phosphatase enzymes have been shown to have important interaction with connexins.

FUNCTIONS OF CONNEXINS

- They can be broadly classified as:
  - *Gap junction-dependent functions*
  - *Hemichannel-dependent functions*
  - *Gap junction and hemichannel-independent functions*

Gap Junction-Dependent Functions

- Gap junction intercellular communication (GJIC) is a central conduit of ions, essential metabolites, and second messengers like Ca^{2+}, cAMP, cGMP and IP between adjacent cells.
- It plays an active role in intercellular signaling and communication.
- Gap junction channels, alternate between "closed" and "open" conformations.
- This is regulated by the following mechanisms:
  - Calcium concentration
  - pH
  - Trans-junctional potential
  - Protein phosphorylation.
- The regulatory sites that are responsive to pH levels are found in the intracellular loop and C terminus domains of the connexin proteins.

- These regions show a little sequence homology between different connexins.
- Hence, differential composition of the gap junction channels determine response to pH in different cell types.
- **Transmembrane voltages:** Large transjunctional voltages close the channels, possibly through the C-terminus interacting with the pore of the channel.
- Phosphorylation status changes the number of gap junctions at the cell–cell interface.
- This happens either through controlling connexin trafficking and degradation or by completely closing or opening the channel for passage of molecules.
- Gap junction are involved in developmental and regulatory events such as embryonic growth, bone modeling, alveolar differentiation, CNS signaling and neural functions.
- Proper gap junction intercellular communication is essential for differentiation of various tissues, including the mammary gland, lens, bone marrow, Sertoli cells, adipocytes, and other cells and organs.
- Restoration of gap junctional communication in colon cancer cells leads to re-establishment of a differentiation phenotype.
- The tumor cells show increased levels of connexin 43 protein and its phosphorylation status.
- Gap junctions also have a role in cell death.
- **Bystander effect,** i.e. gap junctions spread a death signal between dying cells and those adjoining them, which is mediated by calcium influx between these cells.
- For example, spread of injury in the brain following hypoxia/ischemia.
- GJ have also been shown to "rescue" dying cells.
- It is achieved by ensuring passage of substrates like ATP, glucose, ascorbic acid, etc. from one cell to other.
- They also inhibit the passage of cytotoxic agents such as nitric oxide and others.

Hemichannel-dependent Functions

- Involved in various aspects of cell life, including calcium signaling, cell proliferation and death, as well as the normal functioning and development of various cell types.
- Osmoregulatory role in ventricular myocytes.

Gap Junction and Hemichannel-independent Functions

- Connexins also play various roles independent of their channel-forming properties.
- These are mediated through their multiple interacting partners.
- Subsequently leading to the modulation of gene expression resulting in a wide range of effects.
- This is particularly important with respect to their role in tumor biology.

ROLE IN TUMOR BIOLOGY

- Connexins are implicated in the carcinogenic process.
- Disruption of normal connexin functions is a hallmark of many tumors.
- Alteration of connexin expression (over-expression/deletion) leads to changes in gene expression in multiple pathways and cellular functions.
- Cx26, involved in tumor suppression, inhibits cell migration and invasion in a gap-junction independent mechanism in the MDA-MB435 tumor cell line through regulation of β1-integrin and MMP levels.

- It also reverses the malignant phenotype of MCF-7 breast cancer cells.
- Breast tumor cell lines exhibit down-regulation of connexin gene expression.
- Deficiency of Cx43 gap junction is an independent adverse marker for breast cancer.
- Glioblastoma (GBM) with high mitotic index have been shown to have a reduced expression of Cx43.
- Cx32 suppresses growth, invasion, and metastasis of RCC cell lines.
- It is done through various modulators like tight junction proteins, VEGF, etc.
- Controlled expression of Cx43 prevented cell growth independent of its channel forming properties.
- This is through the association of its C-terminal domain with proteins such as ZO-1.
- Alteration of connexin expression that is over-expression/suppression leads to changes in gene expression in multiple pathways and cellular functions as follows:
  - Transcription
  - Metabolism
  - Cell/cell and cell/ECM adhesion
  - Cellular signaling
  - Transport
  - Cell cycle and division.
- One such mechanism is through connexin-responsive elements (CxRE).
- Gap junctional communication induces differential recruitment of sp1 and sp3 transcription factors to the CxRE through the PI3K pathway.
- This regulates expression of genes having this promoter element.
- This effect of connexins on gene expression has also been observed in many studies showing re-expressing connexons in connexin-deficient tumors.
- This affects growth and tumorigenicity of the tumor cells via gap junction-dependent and independent mechanisms.
- It includes regulation of gene expression of proteins involved in the various processes, such as MMPs and TIMPs, SKP2 and p21.
- However, in metastasis, heterocellular gap junctions between tumor cells and cells of the secondary tumor site or lymph nodes increase possibility of metastasis.
- For example, expression of Cx26 and Cx43 is increased in breast cancers with lymph node metastasis as compared to primary tumor confined to the breast.
- Cx43-mediated GJIC results in increased diapedesis of the breast tumor cell line HBL100.
- Heterocellular gap junctions between tumor cells and cells of the secondary tumor site or lymph nodes lead to increased possibility of metastasis.
- Other studies have observed that re-expression of homocellular connexions in metastatic tumor cells results in decreased tumor metastatic potential.
- Suppression of MDA-MB-435 tumor cell line metastatic potential results in increased homocellular GJIC.
- There is also a change in the expressed connexion profile.
- Untreated cells expressed only Cx32.
- Metastasis-suppressed cells expressed Cx43.
- Breast cancer and melanoma cells utilize connexions (Cx43, Cx26) to initiate brain metastatic lesion.
- Activation of twist metastatic gene in breast cancer cells increases Cx43 expression which helps in extravasation, blood vessel co-option and colonization of cancer cells.

- This phenomenon is reversed in hepatocellular carcinoma cells where Cx43 enhances tumor cell malignancy through inhibition of Cx32-mediated GJIC and suppression of Cx32 expression altogether.
- Therefore, the impact of GJIC on tumor metastasis may be dependent on the type of connexion expressed and the type of cells involved.
- The roles of various connexins in cancer is summarized in Table 7.1.

Table 7.1: Important connexins and their role in cancer

Connexin	*Cancer*	*Function*	*Tumor activity*
Connexin 25	Leukemia	GJIC	Pro-tumorigenic
Connexin 26	Breast, cervical	GJIC	Anti-tumorigenic
	Cervical	Hemichannel activity	Anti-tumorigenic
	Breast	Protein-connexin interaction	Pro-tumorigenic
Connexin 30	Glioma, gastric	GJIC	Anti-tumorigenic
Connexin 31.1	Head and neck squamous cell carcinoma	GJIC	Anti-tumorigenic
	Non-small cell lung cancer	Protein-connexin interaction	Anti-tumorigenic
Connexin 32	Breast	GJIC	Pro-tumorigenic
	Renal cell carcinoma, ovarian	Protein-connexin interaction	Anti-tumorigenic
Connexin 36	Cervical	Protein-connexin interaction	Pro-tumorigenic
Connexin 37	Liver, insulinoma	Protein-connexin interaction	Pro-tumorigenic anti-tumorigenic
Connexin 43	Brain	GJIC	Pro-tumorigenic
	Breast	Hemichannel activity	Anti-tumorigenic
	Ovarian	Protein-connexin interaction	Anti-tumorigenic
Connexin 46	Brain	GJIC	Pro-tumorigenic
Connexin 50	Cervical	GJIC	Pro-tumorigenic

POINTS TO REMEMBER

- Gap junctional intercellular communication (GJIC) is a form of cell–cell communication mediating the exchange of small molecules between neighboring cells.
- Gap junctions (GJs) are formed by connexins (Cxs), and are subject to tight and dynamic regulation.
- They are involved in the cell cycle, differentiation, and cell signaling.
- The loss of Cxs and GJs is a hallmark of carcinogenesis, while their induction in cancer cells leads to a reversal of the cancer phenotype, induction of differentiation, and regulation of cell growth.
- An overexpression in heterocellular gap junction has been shown to have a role in tumor metastasis.
- They may also serve as novel targets for anti-tumor therapy.

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Lymphoma Microenvironment and Immunotherapy

8

Ravi Pratap Singh

INTRODUCTION

- Lymphoma microenvironment is a dynamic and interactive supporting network of immune cells, stromal cells, cytokines, blood vessels and extracellular matrix.
- Composition of lymphoma microenvironment is guided by neoplastic cells. The microenvironment influences tumor initiation, progression and drug resistance.

COMPONENTS OF LYMPHOMA MICROENVIRONMENT

- **Immune cells**
  - Cytotoxic T cells (CTLs)
  - Follicular B helper T cells (TFH)
  - Regulatory T cells (Tregs)
  - Natural killer cells (NK)
  - Bystander B cells
- **Stromal cells**
  - Mesenchymal stromal cells (MSCs)
  - Lymphoma associated macrophages (LAMs)
  - Myeloid-derived suppressor cells (MDSCs)
  - Dendritic cells
- **Extracellular components**
  - Extracellular matrix (ECM)
  - Cytokines/chemokines
  - Lymphoma exosome.

ANGIOGENESIS

Mesenchymal Stromal Cells (MSCs)

- MSCs have both anti-inflammatory as well as immunosuppressive properties.
- MSCs differentiate into the fibroblastic reticular cells and follicular dendritic cells necessary for the infiltration of follicular lymphoma in the bone marrow.

MSCs in Lymphomas

- Marrow MSCs from patients with follicular lymphoma overexpress chemokine (C-C motif) ligand 2 (CCL2) resulting in growth sustenance of malignant B cells.

Research and Studies

- Coinjection of MSCs with neoplastic B cells promotes B cell lymphoma growth in the lacrimal glands of immunocompetent mice and are found to be associated with marked increase in CD4+ forkhead box P3 (FoxP3) + T cells and myeloid-derived suppressor cells.

LYMPHOMA-ASSOCIATED MACROPHAGES (LAMs)

LAMs in Hodgkin Lymphoma

- In advanced stage classic Hodgkin lymphoma (CHL) as well as in meta-analyses, a high-density of LAMs is a strong predictor of adverse outcomes in adult patients.
- In CHL, an **increased number of CD68 +** LAMs result in:
  - *Shorter progression-free survival*
  - *Increased likelihood of relapse after stem cell transplantation*
  - *Overall shortened disease-specific survival.*

Therapeutic Developments

- Immunomodulatory drugs such as pomalidomide convert the polarization status of macrophages from M2 to M1 in mouse models of central nervous system (CNS) lymphoma.
- This reduces signal transducer and activator of transcription (STAT) 6 signaling while enhancing STAT1 signaling resulting in increased phagocytic activity of macrophages.
- LAMs are a potential stratification biomarker.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

- Tumors recruit MDSCs by secreting factors like granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and interferon gamma (IFN-γ).
- MDSCs suppress immune surveillance, particularly in the bone marrow.
- MDSCs have been shown to form mature osteoclasts in response to nuclear factor KB ligand (RANKL), increasing bone resorption.
- All these changes thereby influence the ability of tumors to spread into the marrow niche.

MDSCs in Hodgkin Lymphomas

- In CHL at initial diagnosis, all subsets of MDSCs were higher compared to controls. While the patients undergoing therapy showed decreased MDSC subsets.
- Patients with complete response had lower CD34+ MDSCs, monocytic MDSC and polymorphonuclear MDSCs
- Non-responders are seen to have higher levels.

MDSCs in Non-Hodgkin Lymphomas

- CD14 positive monocytes in patients with NHL showed reduced HLA-DR expression.
- This is associated with decreased immune function and more aggressive lymphoma.

MDSCs in Diffuse Large B Cell Lymphoma (DLBCL)

- Patients with DLBCL have higher circulating CD14+ HLA-DR monocytic MDSCs.

MDSCs in Extranodal NK/T Cell Lymphoma

- Study conducted in 32 extranodal NK/T cell lymphoma patients showed higher levels of CD33+ CD11b+ HLA-DR– MDSCs.
- These MDSCs had increased expression of IL-17, arginase-1 and cytokine-inducible nitric oxide synthase (iNOS) and suppressed T cell proliferation.

MDSCs in Cutaneous T Cell Lymphoma

- In cutaneous T cell lymphomas it has been observed that MDSCs and tumor cells inhibit T cell proliferation and promote regulatory FoxP3+ T cells with the expression of programmed death ligand 1 (PD-L1).

Prognosis

- Level of these MDSCs correlates directly with worse clinical prognosis and regulatory T cells (Tregs) proliferation
- The higher levels of MDSCs correlate with shorter progression-free and overall survival.

Research and Studies

- Normal peripheral blood mononuclear cells were incubated with monocytes from patients with B cell non-Hodgkin lymphoma (NHL).
- This resulted in a reduction in T cell proliferation as well as decreased Th1-response observed via measurement of IFN-γ production.
- Anti-CD14 immunomagnetic beads when used to decrease the monocyte population resulted in restored T cell proliferation.

DENDRITIC CELLS

- Dendritic cells aid in the activation of cytotoxic T cells and naive helper T cells.
- Direct follicular dendritic cell contact with the neoplastic cells causes upregulation of microRNA-181a (miR-181a) and reduced levels of proapoptotic Bcl-2-like protein 11 (Bim), resulting in an overall protection from apoptosis.

Dendritic Cells in Follicular Lymphoma

- In follicular lymphoma, tumors with gene expression signatures of dendritic cells and monocytes are shown to be associated with poor outcomes.
- Whereas gene expression signatures of T cell markers and macrophages were associated with prolonged survival.

Research and Studies

- *In vitro* studies were initially promising when DCs were pulsed with either tumor antigen or whole tumor lysate to stimulate immune responses from T cells, however, *in vivo* studies on hematologic malignancies have not demonstrated durable responses.

CHEMOKINES AND CYTOKINES

Role of Chemokines and Cytokines in Classical Hodgkin Lymphoma

- The tumor microenvironment of CHL (constituting 99% of the tumor) is composed of:
  - B cells
  - T cells
  - Eosinophils
  - Plasma cells
  - Neutrophils
  - Macrophages
  - Dendritic cells
  - Fibroblasts.
- All of the above cells are derived from the dysregulated chemokine secretion by the HRS cells.
- Key cytokines playing an active role in the process include:
  - IL-7
  - IL-10
  - TGF-β
  - Chemokine ligand 5 (CCL 5)
  - Chemokine ligand 1 (CCL1)
  - Galectin-1
- T cells surrounding Reed-Sternberg cells express CCL5, which acts as a chemo-attractant for monocytes, eosinophils, basophils and mast cells as well as CD4 positive T cells.
- C-C chemokine receptor type 3 (CCR3) + Th2 cells and eosinophils are attracted by the CCL1(eotaxin) produced by fibroblasts surrounding RS cells.

CYTOTOXIC T CELLS (CTLs)

- During chronic antigen stimulation, lymphocyte activation gene-3 (LAG-3) protein is upregulated on T cells which suppresses CD4+ T cell expansion in response to antigen as well as CD8+ T cell function.
- Specifically, LAG-3 has been shown to maintain tolerance to tumor antigens via its effects on CD8+ T cells.
- In murine models, LAG-3 blockade increases proliferation and effector function of antigen-specific CD8+ T cells within organs and tumors that express their cognate antigen.
- The cytotoxic activity of T cells is enhanced by the targeting of the PD-1 pathway, which can lead to tumor cell lysis.
- Tumor specific activated T cells as well as regulatory T cells express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which binds to CD80/CD86 on antigen

presenting cells and leads to T cell anergy by competing with CD28 as a costimulatory molecule.

CTLs in Lymphoma

- Increased numbers of infiltrating CD8 positive T cells, many expressing cytotoxic markers like TIA-1 have been associated with better outcomes in B cell lymphomas.
- Elevated numbers of cytotoxic lymphocytes positive for programmed cell death-1 (PD-1) was also found to be associated with favorable prognosis in the setting of follicular lymphoma.

REGULATORY T CELLS (TREGs)

- Thymic derived Tregs are involved in preventing autoimmunity.
- Peripheral Tregs maintain tolerance in mucosal sites.
- Both the naturally occurring CD25+ CD4+ Treg populations express FoxP3, which is a more specific marker for regulatory T cells than CD25, CD45RB, or CTLA-4.
- Tregs suppress the activity of bystander T cells, natural killer cells and B cells via CTLA-4, IL-10, and TGF-β1.

Tregs in Inflammation

- FoxP3+ Tregs, particularly in inflamed tissues, express T cell immunoglobulin and mucin-domain containing-3 (TIM-3) which enhances their regulatory function.
- TIM- 3 works as a co-inhibitory receptor that is also expressed on IFN-γ producing T cells, macrophages and dendritic cells, where it leads to inhibition of normal Th1 responses.
- Blockade of TIM-3 signaling appears to demonstrate therapeutic benefit in preclinical tumor models.

Tregs in Lymphoma

- In certain NHL where Tregs are overrepresented in biopsy specimens compared to normal lymphoid tissue, these cells appeared to be recruited by malignant B cells.
- In a study of 280 CHL patients, higher numbers of intratumoral Tregs were associated with better failure free survival and also somewhat better overall survival.
- Similarly, in follicular lymphoma and germinal center subtype diffuse large B cell lymphomas, a positive correlation between disease-specific survival and numbers of intratumoral FoxP3 positive cells was observed.

FOLLICULAR B HELPER T CELLS (T_{FH})

- In the normal germinal center, T_{FH} cells appear to be involved in CD40-mediated interactions in the germinal center.
- Circulating *CD4+ C-X-C chemokine receptor type 5 (CXCR5) + T cells* serve as the memory compartment of T_{FH} cells.
- CXCR5 is the receptor for chemokine ligand 13 (CXCL13), produced by follicular dendritic cells which promotes the entry of B cells into germinal center.
- Hence, the upregulated expression of CXCR5 facilitates contact between the B cells and T cells

T_{FH} in Follicular Lymphoma

- T_{FH} cells are abundant in follicular lymphomas.
- In follicular lymphoma, these cells appear to provide IL-4 stimulation to the B cells and in conjunction with CD40 interactions, aid in the proliferation of neoplastic cells through STAT5 signaling.

T_{FH} in Other Lymphomas

- In patients with low-grade B cell lymphomas like follicular lymphoma or marginal zone lymphomas, circulating T_{FH} cells show reduced C-C chemokine receptor type 6 (CCR6) and increased PD-1.
- Increased levels of PD-1 receptor have also been found in T cells from chronic lymphocytic leukemia (CLL) patients.

NATURAL KILLER (NK) CELLS

- NK cells are CD16+ CD56+ cytotoxic lymphocytes of the innate immune system, which induce apoptosis even in the absence of antibodies and major histocompatibility complex.
- NK cells can recognize tumor antigens via killer-cell immunoglobulin-like receptors (KIRs).
- A subset of NK cells produces IFN-γ, TNF-α, IL-10, and certain chemokines that aid in the differentiation of T cells and dendritic cells.
- Once a tumor microenvironment is developed, TGF-β is induced and TIM-3 expression on NK cells is upregulated.
- Increased TIM-3 expression has been associated with lower NK-cell cytotoxicity and poor outcomes in a variety of neoplasms.

Role of NK Cells in Lymphoma and Therapeutic Potential

- Recent studies have shown significant reduction in NKG2D expression as well as weak cytotoxic activity in NK cells in untreated CHL patients.
- Reactivation of silenced NK cells in CHL is a potential therapeutic target and is being currently pursued. Immune checkpoint inhibitors, like Nivolumab, are being used to recover cytotoxic activity of NK cells in CHL by PD-1 inhibition.
- Drugs targeting heat shock protein-90 have been found to be effective in preclinical studies.

Newer Findings

- In a recent phase 1 study, the bispecific (CD30/CD16a), tetravalent antibody, AFM13 has proven significantly effective in NK cell activation.
- Studies have shown defective NK cell cytolytic function in CLL which is associated with increased cancer risk.
- In mouse models, IFN-γ and perforin protein knockouts develop B cell lymphomas that show suggestion of immunosurveillance defect.

BYSTANDER B CELLS

- Bystander CD 20+ B cells are more numerous in lymphocyte predominant Hodgkin lymphoma (LP-HL) compared with CHL.
- B cell production of IL-10 may aid in antitumor immunosuppression by T cell inhibition.
- Whereas competition with tumor cells (H-RS) for T cell derived survival signals may halt tumor cell growth.

LYMPHOMA EXOSOME

- Exosomes are microparticles that can be secreted by cells and usually range in size from 30 to 100 nm.
- These were thought to be cellular waste, but are now known as carriers of signaling molecules.
- CLL-derived exosomes were shown to induce stromal cells to take on a cancer-associated fibroblast (CAF) phenotype *in vitro*.
- The CAFs, in turn, support a niche that promotes CLL cell adhesion, survival and growth *in vivo*.
- Exosomes produced by lymphoma B cells carrying mutated *MYD88* were reported to reprogram the marrow microenvironment such that mast cells and macrophages were induced to promote endogenous proinflammatory signaling pathways.

EXTRACELLULAR MATRIX (ECM)

- Extracellular matrix facilitates the creation of tumorigenic microenvironment by promotion of angiogenesis and inflammation.
- In solid organ tumors, dysregulated ECM has been shown to expedite cancer progression directly by the following mechanisms:
  - Directly affecting cancer cells causing cellular transformation.
  - Cancer stem cell expansion and
  - Disruption of tissue polarity leading to tumor invasion and metastasis.

ANGIOGENESIS

- Lymphoma tumor microenvironment also includes a rich scaffold of vessels that supply nutrients to the proliferating cells.
- Platelet-derived growth factor (PDGF) recruits PDGF receptor-expressing pericytes to neovessels, thus promoting vascular maturation and stabilization.
- PDGF is also involved in the expression of other stromal angiogenic factors like basic fibroblast growth factor and VEGF.
- Inhibition of platelet-derived growth factor receptor B (PDGFRB) with imatinib mesylate or sunitinib malate has shown some efficacy in carcinoma models.
- One study showed impaired growth of lymphoma in both human xenograft and mouse allograft models with the use of imatinib, a tyrosine kinase inhibitor of PDGFRB.
- Imatinib induces apoptosis of tumor associated PDGFRB positive pericytes and loss of perivascular integrity.

- The tumor endothelium has also been shown to prevent T cell homing, and hence, can serve as a barrier against immunotherapy.
- An inhibitor for endothelin B receptor was shown to increase the adhesion of T cells *in vitro* to human endothelium.
- Inhibitory enzymes, like indolediamine oxidase (IDO), and phenylalanine oxidase interleukin 4-induced gene 1 (IL4I1).
- IL4, secreted by lymphoma associated macrophages and some B-NHL cells
- Immune suppression by Treg expansion and inhibition of effector T cell proliferation and activity.
- Increased expression of FAS ligand (FASL) by NHL B cell induces cytotoxic T cell apoptosis, whereas
- IL-12 secretion induces T cell exhaustion by LAG-3 and TIM-3 induction immunotherapy.

MECHANISMS OF TUMOR MICROENVIRONMENT MEDIATED IMMUNE EVASION AND TUMOR PROGRESSION IN NHL

- A number of mechanisms are implicated in tumor immune evasion and progression.
  - Reduced tumor immunogenicity and immune evasion
  - Loss of lymphoma cell surface molecules/ markers
  - Genetic alterations leading to loss of MHC class I, MHC class II, and CD58 contribute to the failure of CD8+ T lymphocyte, CD4+ lymphocyte, and NK cell-mediated tumor cytotoxicity
  - Overexpression of inhibitory lymphoma cell surface molecules, like PD-L1 and herpesvirus entry mediator (HVEM), which on interaction with their counterparts on T cells lead to impaired T/NK cell activity.
  - Overexpression of CD47 and SIRP-alpha is a lymphoma cell mechanism to evade macrophage-mediated destruction.

Types of Immunotherapy

- Monoclonal antibodies
- Immunomodulating drugs
- Immune checkpoint inhibitors
- CAR T cell therapy.

Monoclonal Antibodies

- They keep cancer cells from growing by blocking signals sent out by the cancer cells.
- They can also bind to the cancer cells and trigger the immune system to kill them.
- Monoclonal antibodies can be attached to toxins, chemo, or radioactive substances.
- Examples:
  - Rituximab: Anti-CD20
  - Brentuximab: Anti-CD30
  - Alemtuzumab: Anti-CD52
- **Follicular lymphoma**
  - Rituximab + chemo
- **Mantle cell lymphomas**
  - Rituximab for maintenance, induction and relapse.

- **Diffuse large B cell lymphoma**
  - Rituximab
  - For relapse: Pembrolizumab
- **T cell Lymphoma**
  - If non-responsive to chemo alemtuzumab/brentuximab.

CHIMERIC ANTIGEN RECEPTOR CAR-T CELL THERAPY

- Immune cells called T cells are removed from the patient's blood.
- They are altered in the lab to have specific receptors (called *chimeric antigen receptors*, or CARs) on their surface.
- These receptors can attach to proteins on the surface of lymphoma cells.
- The T cells are then multiplied in the lab and given back into the patient's blood, where they can seek out the lymphoma cells and launch a precise immune attack against them.
- **Most CAR T cell therapies are still being studied and only available in clinical trials.**
- **Axicabtagene ciloleucel (Yescarta)** approved by the USFDA for:
  - Diffuse large B cell lymphoma
  - Primary mediastinal large B cell lymphoma
  - High grade B cell lymphoma
  - Diffuse large B cell lymphoma arising from follicular lymphoma
- **Tisagenlecleucel (Kymriah)**
  - Diffuse large B cell lymphoma
  - High grade B cell lymphoma
  - Diffuse large B cell lymphoma arising from follicular lymphoma.

POINTS TO REMEMBER

- Composition of the microenvironment plays a vital role in various processes, including the progression, drug resistance and prognosis of lymphoma.
- Targeting microenvironment components is expected to provide novel insights for the precise treatment of lymphoma.
- Nevertheless, there are still many unresolved issues, such as safety, efficacy, drug resistance and the feasibility of drug combination.
- Further studies are warranted to verify and promote the clinical applications of microenvironment-based targeted therapy.
- A deeper understanding of the contribution of microenvironment to lymphomas will help us provide patients with more feasible and effective treatment strategies.

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PD-1/PD-L1 Pathway in Carcinogenesis

9

Naveen Kumar

INTRODUCTION

Programmed Cell Death Protein 1/ CD279.

- The PD-1 protein in humans is encoded by the PDCD1 gene.
- It is expressed on T cells, B cells, monocytes, natural killer cells, dendritic cells and many tumor-infiltrating lymphocytes (TILs).
- It plays an important role in downregulating the immune system and promoting self-tolerance by suppressing T cell activity.
- PD-1 binds two ligands, PD-L1 and PD-L2.
- PD-L1 (CD274) and PD-L2 (CD273) are both coinhibitory.
- PD-L1 is expressed on resting T cells, B cells, dendritic cells, macrophage, vascular endothelial cells and pancreatic islet cells.
- PD-L2 expression is seen on macrophages and dendritic cells alone.
- PD-1 is an immune checkpoint.
- PD-1/PD-L1 interaction ensures that the immune system is activated only at the appropriate time in order to minimize the possibility of chronic autoimmune inflammation.
- It guards against autoimmunity through a dual mechanism of promoting apoptosis (programmed cell death) in antigen-specific T cells in lymph nodes while simultaneously reducing apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells).

MECHANISM OF PD-1/PD-L1 SIGNALING

- In the tumor microenvironment, PD-1 and its ligand PD-L1 perform a vital role in tumor progression and survival by escaping tumor neutralizing immune surveillance.
- Engagement of PD-L1 with PD-1 of T cell creates T cell dysfunction, exhaustion, neutralization, and production in a tumor mass.
- B7-1 (CD80) is a protein expressed on activated T cells and APCs which interacts with the PD-L1 of tumor cells.
- This causes negative regulation of effector T cell activation.
- The function of a tumor overexpressing PD-L1 is to protect itself from cytotoxic T cell mediated cell killing.

- Another subtype of T cells, such as regulatory T cells (Treg, CD4+) create a highly immunosuppressive tumor environment.
- This is done by maintaining the expression of PD-1 on cell surface.
- PD-1 increases the *de novo* transformation of naive CD4+ T cells to Treg cells, thus attenuating immune responses.
- PD-L1 is expressed in a wide range of hematopoietic and non-hematopoietic cells.
- PD-L2 has restricted expression on macrophages, dendritic cells (DCs) and mast cells in the secretion of IL-4 and IFN–γ.
- It has been recently reported that PD-L2 interacts with repulsive guidance molecule B (RGMB) of macrophage (MΦ) proteins.
- Although, there are several reports on PD-L2, little information is available about its role in cancer immunosuppression.
- PD-L1 is responsible for tumor immune modulation.
- The binding affinity of PD-1 with PD-L1 is three times greater than the affinity between PD-1 for PD-L2.
- PD-L1 expressions in tumor cells and hematopoietic cells are determined by the stimulation of pro-inflammatory cytokines such as IFN-γ and TNF-α.
- PD-L1 of tumor stromal components, such as fibroblast, extracellular matrix (ECM), tumor-associated macrophages (TAM), and myeloid derived suppressor cells (MDSC) deactivate T cell (CD8+) mediated cancer cells killing through interaction with PD-1.
- Similarly, maturation of MDSC to TAM and secretion of pro-inflammatory cytokines (IFN-γ) from TAM suppress T cell functions.
- This provides positive modulation of PD-1 and PD-L1 interaction.

Mechanism of PD-1/PD-L1 Mediated Immune Resistance

- PD-1-associated immune-resistance depends on the accessibility of PD-L1 ligand in the tumor.
- There are two types of immune resistance:
  1. Innate immune resistance
  2. Adaptive immune resistance

Innate Immune Resistance

- In glioblastomas PD-L1 expression is by downregulation of PTEN which is linked to activation of PI3K-Akt tumorigenic signaling.
- In lymphomas and lung cancers PD-L1 expression occurs through upregulation of the STAT3 and lymphoma kinase (ALK) signaling resistance.
- The STAT3 activation is modulated through IL-6 and the IL-6-STAT3 axis is considered as one of the crucial pathways in tumorigenic immune suppression.

Adaptive Immune Resistance

- PD-L1 expression is induced due to the secretion of pro-inflammatory IFN-γ from tumor and tumor-stromal cells that neutralize CD8+ cytotoxic T cell-induced anti-tumor immune responses.
- Brandon et al investigated the clinicopathologic characteristics of non-small cell lung cancer (NSCLC) subsets defined by PD-L1 expression in either tumor cells or tumor-infiltrating immune cells.

- PD-L1 expression by IHC is currently the only available predictive biomarker for NSCLC response to immune checkpoint blockade.
- PD-L1 expression in either tumor cells or tumor-infiltrating immune cells is correlated with high histologic grade and solid subtype.

PD-1/PD-L1 in Hematological Malignancies

- Among hematological malignancies, PD-1/PD-L1 inhibitors have been successful, so far, only in the treatment of classical Hodgkin lymphoma.
- Typically exhibits an over-expression of PD-1 ligands.
- It is due to alterations in chromosome 9p24.1 because the locus contains the genes encoding PD-L1 and JAK2.
- PD-L1 and PD-L2 are expressed on the surface of malignant cells in 65–100% of classical Hodgkin's lymphoma and in 54% of nodular lymphocyte predominant Hodgkin's lymphoma.
- This results in increased expression of proteins on RS cells.
- JAK2–STAT signaling promotes further increased expression of PD-L1 by augmenting transcription of the PD-L1 gene.

STRATEGIES TO MEASURE PD-L1/PD-1 EXPRESSION

- Immunohistochemistry is one potential biomarker, however, yet to be standardized.
- Challenges regarding standardization are as follows:
  - Use of different monoclonal antibody clones.
  - Heterogeneous PD-L1 expression in different regions of the same tumor specimen.
  - Absence of PD-L1 expression on small biopsies may not reflect the systemic immunologic landscape.
- Other methods like flow cytometry which has an added advantage of simultaneously measuring the expression of PD-1 and PD-L1 in malignant cells and immune cells.
- Immunomagnetic selection and cell track analyzer.
- ELISA, FISH probe specifically targeting the gene locus encoding PD-L1 located at 9p24.1
- The incidence of 9p24.1 amplification (at least >3 copies) is seen in early-stage HL-24% and in advanced stage HL-50%.
- Patients with HL harboring 9p24.1 amplifications had a shorter progression-free survival.
- FISH studies promise to be another potential method for predicting the response to PD-1/PD-L1 treatment.

PD-1/PD-L1 Inhibition and Uses

- Monoclonal antibodies are drugs called checkpoint inhibitors.
- They reduce toxicity within tolerable limits.
- Shrink solid tumors.
- Suppress advanced tumors and metastasis.
- Overall improve patient survival.
- Inhibitors will cause resurrection of T cell mediated anti-tumor immune effect.

Targeted Therapy

- **PD-1 inhibitors:** Nivolumab and Pembrolizumab.
- Nivolumab has been approved by USFDA in treatment of advanced and metastatic cases of melanoma, head and neck squamous cell carcinoma, non-small cell lung cancer and renal cell carcinoma.
- Pembrolizumab has been approved by USFDA for treatment of recurrent and metastatic cases of head and neck squamous cell carcinoma.

PD-L1 Inhibitor

- Atezolizumab has been approved by USFDA in the treatment of non-small cell lung cancer and in the treatment of urothelial carcinoma.

PD-1 INHIBITORS FOR COMBINATION THERAPY

- Monotherapeutics have been demonstrated to be a successful immunotherapy regimen.
- Because of safety and better clinical activity of monotherapy the field is moving toward the direction of discovering novel combination therapies.
- Various anti-cancer agents are used in combination with PD-1 antibody inhibitors.

Combination of PD-1 and Kinases Inhibitors

- Mitogen-activated protein kinase is an effective regulator of BRAF mutation which is responsible for the metastasis of cutaneous melanoma.
- The mutation of BRAF has also increased the expression of the PD-1 and PDL-1 molecule.
- It induces potential drug resistance with the involvement of tumor stromal cells.
- The combination of PD1 immunotherapy with a BRAF inhibitor resulted in synergistic anti-tumor response and prominent tumor growth inhibition.

Combination of PD-1 and Other Checkpoint Inhibitors

- For example, Nivolumab and Anti-CTLA-4 antibody- ipilimumab are under clinical trials in treatment of various cancers.
- Various other combinations of PD-1 with VEGF inhibitors and chemotherapeutic drugs are under clinical trials.

Adverse Reactions

- Once the immune checkpoints have been blocked, the equilibrium between the autoimmunity and immune tolerance will be affected.
- Immune-meditated adverse reactions (IMARs) is a term coined to describe the side effects of new immunotherapy.
- Adverse effects like fatigue (32%), rash (23%), skin disorders (36%), GI events (18%), endocrinopathies (13%) and diarrhea (18%).

POINTS TO REMEMBER

- Immune checkpoint mechanisms such as the PD-1/PD-L1 pathway have led to a clinically significant antitumor response.

- Rapid improvement is being demonstrated in the treatment of various cancers which resulted in USFDA approval for these newly discovered agents.
- Currently, multiple studies are undergoing to improve the success rate and more efficient use of these agents at an earlier stage of the disease in single or in combination.
- This can significantly increase overall survival or cure rates.

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10 Techniques to Characterize Foreign Materials in Pathological Specimens

Naveen Kumar

INTRODUCTION

- Pathologists typically encounter many exogenous materials in clinical specimens during routine histopathological examinations.

Foreign Substances

- **Voluntary causes** (e.g. tattoos or cosmetic fillers)
- **Involuntary causes** (e.g. trauma, surgery, and ingestion or inhalation of particulate materials).
- Most are incidental findings seen in histological or cytological material removed for other purpose (e.g. dermal suture granulomas, lubricant in Pap smears).
- Some are removed because they are the cause of an undiagnosed lesion (e.g. dental amalgam tattoo mimicking malignant melanoma).
- They are easily recognized by routine light microscopy, and do not pose a diagnostic problem.
- However, on occasion a full characterization becomes important to rule out other entities (e.g. melanin vs. dental amalgam), and confirm the diagnosis (e.g. cutaneous deposits of silver in argyria).
- Histopathologically, they generate immune, inflammatory, or granulomatous reactions.
- Microscopic examination is not always sufficient to identify the nature of these foreign substances on the basis of characteristic appearance and birefringence patterns.
- They are usually described as particles, pigments, granules, fibers, or fragments of various forms, sizes, and colors.

FOREIGN MATERIALS

Tattoos

- Mechanical introduction of insoluble pigments into the dermis.
- With the increasing incidence of tattooing fashion trend, physicians should be able to recognize tattoo complications.

Tattoo Reactions

- **Inflammatory:** Allergic to salts used, focal oedema, pruritus, papules, or nodules at the tattoo site.
- **Infections:** Bacterial, viral or mycotic.
- **Neoplastic complication** rarely described within tattoo pigments includes keratoacanthoma, squamous cell and basal cell carcinoma, leiomyosarcoma, and melanoma.
- **Microscopically** they localize around vessels in the upper and mid-dermis in macrophages and fibroblasts (Fig. 10.1).
- **Extracellular deposits:** Found between collagen bundles. The pigment is generally refractile but not doubly refractile.

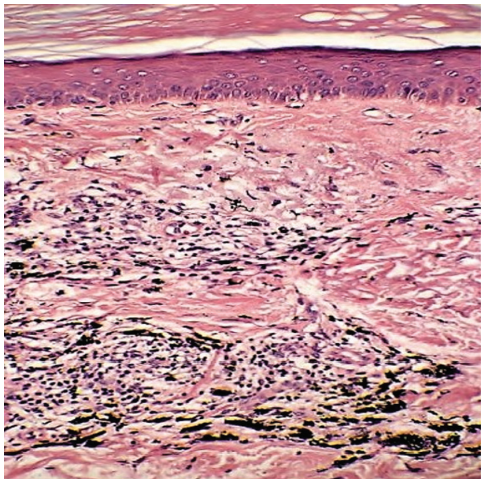

Fig. 10.1: Tattoo pigment with associated inflammatory reaction

Suture

Various types of suture materials are as follows:

- **Common:** Silk, polyvinylidene fluoride (PVDF), polyglycolic acid, and catgut.
- **Absorbable:** Catgut, polydioxanone, polyglycolic acid. They are used for deep tissues, membranes, and subcuticular skin closure.
- **Non-absorbable:** Polypropylene, nylon (Fig. 10.2), stainless steel. They are used for skin (removed) and some deep structures (tendons, vessels, nerve repairs—not removed).

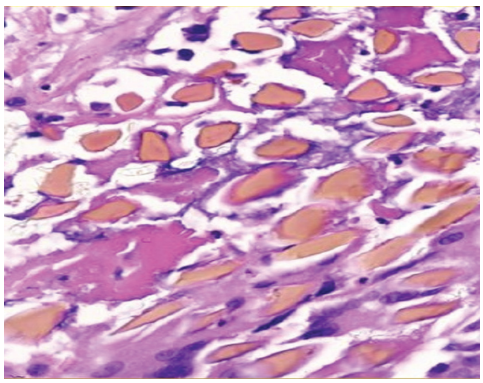

Fig. 10.2: Nylon suture granuloma

Polypropylene

- These are non-absorbable meshes for hernia repair and emergency abdominal wall reconstruction.
- They are colorless rounded structures on H&E and brightly birefringent on polarized light.[2]

Silicone

- Silicone is an organosilicon compound which can be in liquid, gel or solid form.
- A liquid form of silicone is dimethicone (dimethylpolysiloxane).
- **On H&E** they are refractile, colorless, non-birefringent and usually found within phagocyte vacuoles or extracellularly, especially lining partially "washed-out" spaces.

Collagen Implant

- **Natural polymers:** Bovine collagen and nonsulfated glycosaminoglycan, hyaluronic acid.
- The injection of bovine collagen is a relatively safe procedure used to correct defects caused by acne scars, trauma, and aging.

Adverse Reactions

- Erythema
- Urticaria
- Abscess formation
- Induration of the injection site
- Granulomatous reactions.
- There is a theoretical risk of prion transmission when materials of bovine origin are used.
- No such complication has yet been reported.
- On microscopy they are acellular and structureless with diffuse eosinophilic lobules.
- Bovine collagen is non-refringent and stains a pale gray-violet color with Masson's trichrome stain.
- It is absorbed and it can no longer be detected by light microscopy or immunofluorescence techniques after several months.
- The native collagen is different from bovine collagen because it is birefringent and stains green with Masson's trichrome stain.

Hyaluronate

- They present as diffuse nodular lesions in the labial vestibule.
- Represent as hyaluronate dermal fill granulomas that migrated from the original injection sites.
- They are positive for alcian blue and mucicarmine.

Synthetic Fillers

Lactate Polymer

- They are unique and form foreign body granuloma surrounding ***"surf board" shaped large vacuoles that contain "broken glass-like particles".***

Hydroxyethyl Methacrylate

- They form well circumscribed granuloma and show stretched multinucleated giant cells.
- The cleft contents of hydroxyethyl methacrylate are **nonrefractile.**

Cellulose

- Cellulose is present in tissues as cotton, wood splinter, food particles (aspiration), IVDU (microcrystalline cellulose from oral medications).
- It does not stain well with H&E but it is birefringent under polarized light.
- Special stains (GMS, PAS) are also helpful in identification.

Dental Amalgam

- Dental amalgam has multiphasic material containing silver (Ag), tin (Sn), mercury (Hg), and lesser amounts of copper (Cu).
- Incidental tattooing of buccal mucosa may occur during dental procedures (Fig. 10.3).
- Prolonged tissue implantation leads to loss of mercury and tin, and persistence of silver with sulfur (S) and selenium (Se) deposition.

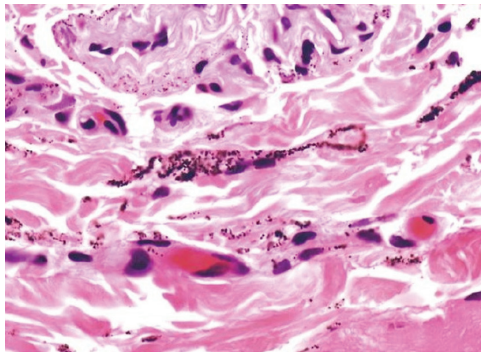

Fig 10.3: Dental amalgam tattoo pigment in sub-epithelium of buccal mucosa

Starch

- Starch powder, a lubricant of surgical gloves, is well recognized as a common contaminant.
- They are refractile, glassy, polygonal bodies, 5–20 micrometer in diameter.
- They often exhibit a ***central dot or "Y"-shaped structure.***
- The ***Maltese cross under polarized light is characteristic,*** but not specific, for starch and can also be seen with some inorganic particles.

Talc

- Talc or hydrated magnesium silicate, is formed during the breakdown of anthrophyllite rock.
- It is used in gloves and as a binder in oral tablets to hold the medication together.
- It is polarizable and birefringent foreign body.

Acrylic Polyamide Plastic Embolization Material

- Embolization microspheres have been developed for tumor embolization and treatment of vascular malformations.
- Uterine artery embolization for treatment of fibroids.
- Several materials have been used like polyvinyl alcohol, collagen, dextran.
- On microscopy they are rounded often folded circular eosinophilic to weakly basophilic objects usually in an intravascular location (Fig. 10.4).
- It is usually mistaken for parasite.
- Mucicarmine, trichrome and Sirius red stains are positive.

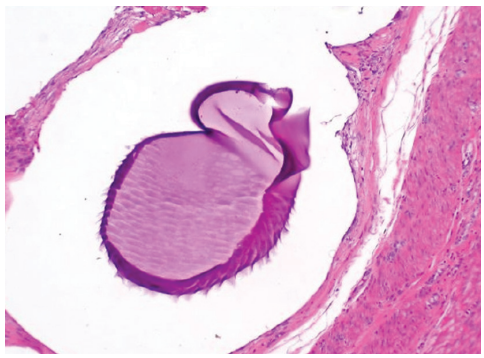

Fig. 10.4: Intravascular acrylic polyamide plastic with Venetian blind artifact mimicking parasite

DRUG DEPOSITS AND PIGMENTATION

Antimalarial Drugs

- The long-term use causes cutaneous pigmentation.
- Yellow pigmentation seen with quinacrine (mepacrine), although the histopathology has not been described.

- Pretibial pigmentation is more common—slate gray to blue-black in color.
- Pigment granules have variable staining characteristics like some staining for hemosiderin, some for melanin, and some for both.
- They can be seen in macrophages as well as extracellularly.

Phenothiazines

- It causes gray-blue pigmentation in sun-exposed areas.
- On microscopy, refractile, golden-brown pigment with staining properties of melanin is found in the dermis along collagen bundles.
- It is seen in macrophages, especially around vessels.
- Electron microscopy shows melanin granules in macrophages and other bodies of varying electron densities that represents metabolites or complexes of the drug.

Tetracycline

- Causes teeth pigmentation.
- Bluish pigmentation of cutaneous osteomas.
- Bluish-green pigmentation in areas of trauma on the lower legs, has resulted from the use of tetracycline.
- Rarely, pigmentation of acne scars on the face occurs.

Minocycline

- Bluish-black pigmentation of scars.
- Blue-gray circumscribed pigmentation of the lower legs and arms.
- They are present in macrophages, perivascular areas and dermal dendrocytes.
- May deposit on elastic fibers or lie free in the dermis.
- Pigment is positive with both the Perls' method for iron and the Masson–Fontana method for melanin.
- It is non-birefringent and nonfluorescent.

Amiodarone

- Slate-gray discoloration of sun-exposed areas.
- On H&E, yellow-brown granules are found in macrophages, which tend to accumulate around blood vessels at the junction of the papillary and reticular dermis.
- Generally believed to be lipofuscin but recent evidence suggest that the material represents amiodarone itself.
- The granules stain positively with the PAS, prolonged Ziehl–Neelsen, Fontana, and Sudan black methods.

OTHER METHODS

- Infrared spectroscopy
- Raman laser spectroscopy
- Scanning electron microscopy with energy dispersive X-ray analysis.

Recent Advance

- Laser-induced breakdown spectroscopy.

Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM/EDXA):

- It was first introduced in the 1960s.
- It has both scanning electron microscopy (SEM) together with energy dispersing microanalyzer (EDX).
- It was used by Raso et al, to examine the bioreactivity of silicone implants.
- It is a method for determining the elemental composition of a particle that can be localized in a tissue section.
- SEM together with EDX are used in toxicology and pathology for determining exogenic and endogenic toxic substances.
- Generally, provides qualitative information.
- Quantitative SEM/EDXA are available particularly for materials for which laboratory standards of known composition are available.

Sample Processing

- Routinely an unstained section of 5 μm on a carbon disc and place the adjacent section from the paraffin block on a glass slide for H&E staining.
- Localize area of interest on H&E and compare to the carbon disc.
- If only an original stained section is available on glass, SEM/EDXA can still be useful.
- Remove coverslip and the background glass examined away from the specimen, will contain elements in glass (usually Si, O, Ca). (Na, Mg, Al, Cl, K, variably present).

Infrared Spectrophotometry

- It became available in 1940s and gives a molecular "fingerprint" that can be compared to reference spectra.
- Unstained section of 5 μm adjacent to H&E stained section or carbon disc section placed on aluminum coated (reflective) glass slide can be used for this.
- Principle –
  Energy of molecule = Electronic energy+ Vibrational energy + Rotational energy.
- When energy in the form of infrared radiation is applied then it causes the vibration between the atoms of the molecules.
- When the applied infrared frequency is equal to natural frequency of vibration then Absorption of IR radiation takes place and a peak is observed.
- Different functional groups absorb characteristic frequencies of IR radiation.
- It gives the characteristic peak value.
- IR spectrum of a chemical substance is a **finger print of a molecule for its identification.**
- IR spectroscopy is concerned with the study of absorption of infrared radiation, which causes vibrational transition in the molecule.
- It is ***Also known as Vibrational spectroscopy.***

Raman Spectroscopy

- Sir Chandrasekhara Venkata Raman who won the Nobel prize in Physics for discovering the 'Raman effect'.
- **Principle:** When monochromatic radiation is incident upon a sample then this light will interact with the sample.
- It may be reflected, absorbed or scattered in some manner.

- It is the scattering of the radiation that occurs which gives information about molecular structure.
- Raman effect is based on scattering.

Recent Advance

Laser-induced Breakdown Spectroscopy (LIBS)

- An all-optical instrument based on laser-induced breakdown spectroscopy (LIBS) has been developed to create multi-elemental images of biological tissues.
- It was primarily developed to visualize the distribution and kinetics of metal nanoparticles in the kidneys of laboratory animals.
- LIBS imaging successfully elucidated the chemical nature and distribution of exogenous agents, such as aluminum (Al) in a granuloma and a pseudolymphoma.
- It also demonstrated Titanium (Ti), copper (Cu), chromium (Cr) and tungsten (W) in lymph nodes and/or the skin.
- **Principle:** A magnification lens is used to focus the laser pulse.
- Then the plasma emission is collected by 2 lenses.
- This is connected to the fiber systems coupled to a spectrometer.
- Spectrometer is equipped with a camera synchronized to the laser.
- It can be performed directly on the paraffin block using Nd:YAG laser.

BACKGROUND ELEMENTS OF TISSUE

- Phosphorus (P) or sodium (Na) is a constitutive element of every cell in tissue.
- This creates elemental images of 'background elements' using LIBS.
- This allows proper visualization of the global architecture of the tissue.

Subcutaneous Nodular Lesions

- Persistent subcutaneous nodular lesions following vaccination or hyposensitization therapy.
- Cutaneous adverse reactions following vaccines or hyposensitization therapy containing Al salts are common.
- Overlapping histopathological patterns mimic the patterns of other conditions such as granuloma, panniculitis, or pseudolymphoma.
- LIBS allows direct *in situ* visualization of the levels of Al in a skin nodule and at the site of hyposensitization therapy.

Pigmented Lymph Nodes

- Pigmentated lymph node is highly suggestive of metastatic malignant melanoma.
- However, exogenous ink particles from tattoos are the most frequent cause.
- This represents a pitfall for surgeons and pathologists during lymph node biopsies or dissection procedures.
- Pigmented lymph nodes pose a diagnostic challenge.
- LIBS has been used to find out the exact element present.
- Elevated levels of titanium (Ti) in lymph nodes that corresponded with the topography of the black pigment reported after histopathological examination. This confirmed the diagnosis of Tattoo pigment.

Role of LIBS

- A need exists for *in situ* elemental imaging of a steadily growing number of identified diseases characterized by a metal imbalance in cells and tissues (e.g. metal overload diseases, neurodegenerative diseases, or even cancers).
- LIBS could provide robust, valuable complementary elemental information to unravel the pathogenesis of such metal-related diseases.
- Combination of standard histochemical procedures with LIBS multielemental imaging is a promising approach for routine investigations of the chemical composition of foreign substances contained in paraffin a embedded specimens.

POINTS TO REMEMBER

- Medical uses of exogenous materials are widespread and will likely increase.
- New medical materials and devices will continue to appear in pathology specimens.
- Familiarity with these materials may help pathologists avoid possible sources of diagnostic error.
- Accurate characterization of these foreign materials can benefit patients, clinicians and regulatory agencies to monitor and change materials that causes harm to patients.

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Section

2

Recent Advances

DNA Microarray

11

Poonam Yadav

INTRODUCTION

- DNA microarray or gene chip is a collection of microscopic DNA spots attached to a solid surface.
- The chip is usually made of glass, nylon, or silicon.
- Each microarray chip is arranged as a checkerboard of 105 or 106 features.
- Each DNA spot contains **picomoles** (10^{-12} moles) of a specific DNA sequence, known as **probes.**
- There are millions of copies of a unique DNA probe (often 25 nucleotides long) in every spot.
- The probe sequences are designed and placed on an array in a regular pattern of spots.
- Fluorescently labeled target sequences that bind to a probe sequence generate a signal.
- Microarray is a 2D array on a solid substrate that assays a large amount of biological material.
- It is a high-throughput, miniaturized and parallel screening method.
- DNA microarray is a genomic approach to studying more than one gene at a time.

Terminology

- **Probe set:** It is a set of probes designed to detect one transcript.
- **Probe cell:** A single square feature on an array containing probes with unique sequences.
- **Mismatch probe (MM):** It is a probe that is complementary to a reference sequence except for a mismatch at a central position.
- **PM:** It is a probe that is a perfect complementary match of the specific gene transcript.
- **Probe pair:** It consists of two probe cells perfect match (PM) along with corresponding mismatch (MM) that are associated together.

Contents of a DNA Microarray

- **cDNA:** "Full-length" cDNA from a gene library
- **Expressed sequence tags (ESTs)** from a gene library

- These are short sub-sequence of a cDNA sequence.
- They are used to identify gene transcripts.
- Each EST is 500 to 800 nucleotides long.

- **Oligos:** Usually 50–70 mers are present. They may be 'spotted' from an oligo set or printed one codon at a time.

HISTORICAL BACKGROUND

- The concept of DNA microarrays began in the mid-1980s.
- It developed from southern blotting.
- Introduction of solid support instead of nylon membranes allowed miniaturization.
- 'Quantitative Monitoring of Gene Expression Patterns with a complementary DNA microarray' was reported by Patrick Brown, Mark Schena and colleagues in Science (1995).
- High-density microarray was invented in 1989 by Stephen PA Fodor and colleagues.

PRINCIPLE

- Core principle is hybridization between two DNA strands.
- Hybridization is the property of complementary nucleic acid sequences to specifically pair with each other.
- A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands.

DNA MICROARRAY PROCEDURE

- It consists of the following steps:
  - Sample collection
  - mRNA isolation
  - Creation of labeled cDNA
  - Hybridization
  - Scanning inside a DNA microarray scanner
  - Data analysis
- mRNA isolation can be done either of the two methods:
  - Liquid-liquid extraction method
  - Column-based extraction method
- The purified RNA is analyzed for:
  - Quality by capillary electrophoresis and
  - Quantity using NanoDrop spectrophotometer.
- The labeled cDNA is generated via reverse transcription.
- The cDNA is then denatured using denaturing agents, for example, sodium hydroxide (NaOH), formamide and dimethyl sulfoxide (DMSO).
- The denatured cDNA is then labeled using dyes, for example, Cy3, Cy5 and biotin.
- In one-channel assays only one dye is used.
- In two-channel assays two dyes are used.
- The labeled samples are then mixed with a hybridization solution.
- The composition of hybridization solution is as follows:
  - **tRNA:** It blocks nonspecific DNA hybridization.

- **Dextran sulfate:** It displaces water and effectively increases the concentration of the probe.
- **Saline sodium citrate:** It controls stringency as sodium ions decrease the electrostatic repulsion of two single strands.
- **Sodium dodecyl sulfate:** Reduces background staining.
- **Heparin:** Decreases background staining and abolishes the binding of proteins.
- A blocking agent (such as COT1 DNA, salmon sperm DNA, calf thymus DNA, PolyA or PolyT).
- **Denhardt's solution** is a mixture of blocking agents used in membrane-based hybridization protocols.
- It ***reduce***s non-specific binding of detection reagents to the northern and southern blot membranes.
- The mixture is then denatured and added to the pinholes of the microarray.
- The holes are sealed and the microarray is hybridized, either in a hybridization oven in which mixing occurs by rotation. Or in a mixer in which mixing occurs by alternating pressure at the pinholes.
- After overnight hybridization, all nonspecific binding is washed off.
- The microarray is then dried and scanned by a machine that uses a laser to excite the dye and measures the emission levels with a detector.
- The image is gridded with a template and the intensities of each feature is quantified.

TYPES OF MICROARRAYS

- **Spotted DNA arrays ("cDNA arrays")**
  - They were developed by Pat Brown at Stanford.
  - PCR products (or long oligos) from known genes usually100 nucleotides in length are spotted on glass, plastic, or nylon support.
  - In spotted microarrays, the probes are oligonucleotides, cDNA, or small fragments of PCR products that correspond to mRNAs are "spotted" onto the chip.
  - Probes are synthesized prior to deposition on the array.
  - A common approach utilizes an array of fine pins or needles controlled by a robotic arm that is dipped into wells containing DNA probes and then depositing each probe at designated locations on the array surface.
  - User can choose the probes to be spotted according to specific experimental needs.
  - These probes receive complementary cDNA or cRNA "targets" derived from experimental or clinical samples.
  - Relatively low-cost microarray that may be customized for each study.
  - Avoids the costs of purchasing often more expensive commercial arrays.
  - Vast numbers of genes on the larger oligonucleotide arrays are usually not of interest to the investigator.
  - They are highly customizable.
- **Oligonucleotide arrays (Affymetrix)**
  - They comprise a small number of 20–25 mers/gene.
  - They are enabled by photolithography from the computer industry.
  - Available off the shelf.

- A solid support contains covalent linker molecules having a protecting group on the free end that can be removed by light.
- UV light is directed through a photolithographic mask.
- This deprotects and activates selected sites with hydroxyl groups.
- Following this coupling occurs with incoming protected nucleotides that attach to the activated sites.
- The process is repeated till the desired oligonucleotide array is ready.
- In oligonucleotide microarrays, the probes are short sequences designed to match parts of the sequence of known or predicted open reading frames.
- They are produced by printing short oligonucleotide sequences designed to represent a single gene by synthesizing this sequence directly onto the array surface instead of depositing intact sequences.
- Sequences may be longer (60-mer probes used in Agilent) or shorter (25-mer probes produced by Affymetrix) depending on the desired purpose.
- Longer probes are more specific to individual target genes.
- Shorter probes may be spotted in higher density across the array and are cheaper to manufacture.

- **Ink-jet microarrays (Agilent)**
  - In this large number of 25–60 mers are "printed" directly on glass
  - The *in situ* synthesis printing process consists of the following steps:
    - Non-contact industrial inkjet printing process
    - Both catalog and custom microarrays can be made.
    - Oligo monomers are deposited uniformly onto specially prepared glass slides, base-by-base, from digital sequence files.
  - Flexible, rapid, but expensive process.
  - A ***web-based application for synthesizing customized microarrays eArray*** is also available.
- The differences between spotted and oligonucleotide arrays are shown in Table 11.1.

Table 11.1: Differences between spotted and oligonucleotide arrays

Spotted arrays	*Oligonucleotide arrays*
Relatively cheap to make (~$10 to make).	Expensive ($500 or more to make).
Flexible—spot anything you want.	Limited types are available, no chance for specialized chips.
Cheap—so the experiment can be repeated many times.	Fewer repeat experiments usually.
Highly variable spot deposition.	More uniform DNA features.
Usually, we have to make our own chip.	We can buy it off the shelf.

APPLICATIONS

- The reasons for using DNA microarray are the following:
  - To observe genomic gains and losses by Microarray Comparative Genomic Hybridization (CGH).

- To observe mutations in DNA.
- Single nucleotide polymorphisms (SNPs) of our genome (Hap Map project).
- To study aberrations in methylation patterns.
- Alterations in gene copy number.
- Pathogen detection.

Gene Expression Analysis

- The process of measuring gene expression via cDNA is called expression analysis.
- It is used to detect DNA or RNA that may or may not be translated into proteins.
- Thousands of genes are simultaneously assessed.
- The effects of certain treatments, diseases, and developmental stages on gene expression are studied.
- For example, microarray-based gene expression profiling can be used to identify changes in gene expression by a pathogen.
- This is achieved by comparing expression in infected to that in uninfected cells or tissues.

Comparative Genomic Hybridization

- It is a molecular cytogenetic method for analyzing copy number variations (CNVs).
- Ploidy level in the DNA of a test sample is compared to a reference sample.
- CGH is only able to detect unbalanced chromosomal abnormalities.
- Array CGH (aCGH) has been developed, allowing for a locus-by-locus measure of CNV with increased resolution.
- In array CGH, the metaphase chromosomes are replaced by cloned DNA fragments.
- This makes it possible to map the changes directly onto the genomic sequence.
- **Prader–Willi syndrome (PWS)** is a paternal structural abnormality involving chromosome **15q11–13.**
- While a maternal aberration in the same region causes **Angelman syndrome (AS).**
- In both syndromes, the majority of cases (75%) are the result of a **3–5 Mb** deletion of the PWS/AS critical region.

Single Nucleotide Polymorphisms

- Components of a SNP array include the following:
  - Allele-specific oligonucleotide (ASO) probes
  - Fluorescent dye-labeled nucleotides
  - A detection system.
- Most important clinical applications of SNP arrays are for determining disease susceptibility.
- SNP-based genetic linkage analysis can be used to map disease loci and determine disease susceptibility genes in individuals.
- The combination of SNP maps and high-density SNP arrays allows SNPs to be used as markers for genetic diseases that have complex traits.

Disease Diagnosis

- DNA microarray has proved to be helpful in investigating various diseases.
- Earlier cancers were classified on the basis of the organs in which the tumors develop.
- Now, classification of the types of cancer is based on the patterns of gene activity in the tumor cells.
- Microarray-generated expression data can be used as a correlate of a particular cell phenotype.
- Specific profiles of gene expression are referred to as "molecular signatures".
- On the basis of expression profile of the marker gene, molecular signatures associated with either a good or poor prognosis generated.
- Using DNA microarray, Martin and colleagues were able to identify 170 genes differentially expressed in invasive breast tumor cells.
- Microarray technology has been utilized in the identification of the following viruses:
  - Avian influenza (H5N1)
  - Viral hemorrhagic fever (Marburg virus)
  - SARS virus, etc.
- **Flu chip:** It is a gene chip that requires only matching sequences from a single gene of the influenza virus.
  - This chip only needs 15 sequences from a single gene to reliably identify avian influenza.
  - It can detect even a few mutations in the genes like hemagglutinin and neuraminidase genes.
- **Greene chip microarray** analysis
  - It is useful in viral hemorrhagic fever like syndrome for its differential diagnosis from malarial cases.

Drug Discovery

- DNA microarray finds extensive application in pharmacogenomics.
- Multistranded DNA and RNA microarrays can be used to identify novel drugs that bind to multistranded nucleic acid sequences.
- This approach can be used to discover new drugs and biologicals that have the ability to inhibit gene expression.
- These microarrays also allow for the characterization of their structure under different environmental conditions.
- **Disease pathway identification**
  - Using genome-wide expression profiling hypotheses are generated for complex disease mechanisms.
  - Possible drug targets in their pathways are identified.
- **Disease pathway validation**
  - This is done by disrupting the pathways that will actually affect the disease etiology.
  - This helps us to study the effects of various factors on gene expression.
- **Compound screening**
  - Mechanism of action and toxicity can be easily studied.

- Small interfering RNA is now being used to rapidly and specifically inhibit gene function.
- These hold promise as novel therapeutic agents of the future.

Toxicological Research

- Genetic impact of toxins on the cells is studied.
- How these effects are passed on to the progeny.
- Gene expression profiling shows that the specific genes are repressed or induced upon exposure to toxic stress in varying amounts depending on the type of toxins and types of cells.

Gene ID

- Small microarrays to check IDs of organisms in food and feed (like GMO).
- Mostly combining PCR and microarray technology.

Nutrigenomic Research

It is the study of variations in the genes related to the influence of diets, for example:

- Effects of calorie restriction on gene expression.
- Obesity and high-fat diets.

POINTS TO REMEMBER

- DNA microarray has a large number of applications in medicine.
- It is presently restricted due to the high cost.
- The major advantages are as follows:
  - Provides data for thousands of genes.
  - Fast and easy to obtain results.
  - Huge step closer to discovering cures for diseases and cancer.
- The major disadvantages are as follows:
  - Very little knowledge is available about many genes
  - DNA array can only detect sequences that the array was designed to detect.
  - Just because mRNA is "turned on" doesn't mean proteins are made
  - Cross-hybridization is possible
  - Data has to be validated by other methods (e.g. qPCR)
  - Weakly expressed genes may not be detected
- However, despite this DNA microarray holds great promise as an important tool in the delivery of personalized medicine.

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Recent Advances in Cervical Cancer Screening

12

Sakshi Sirswal

INTRODUCTION

- Cervical cancer is the leading cancer among Indian females.
- In view of the Indian scenario an effective screening methodology is required.
- Since the introduction of the Pap test, rates of cervical cancer have dramatically declined.

Normal Anatomy

- The cervix, constituting the lower portion of the uterus, is cylindrical or conical in shape and measures 3–4 cm in length and 2.5–3.5 cm in diameter.
- It varies in size and shape depending on the age, parity and hormonal status of the woman.
- The cervix opens into the vagina through the external os. The supravaginal portion meets the body of the uterus at the internal os.
- The portion of the cervix that is exterior to the external os is called the ectocervix. It is readily visible during per-speculum examination.
- The portion above the external os is called the endocervix.
- The endocervical canal, which traverses the endocervix, connects the uterine cavity with the vagina and extends from the internal to the external os.
- Normally, the ectocervix is pink and the endocervix is reddish, granular.

Normal Histology

- The ectocervix is lined by stratified squamous epithelium and endocervix is lined by simple columnar epithelium.
- The stroma of the cervix is composed of dense, fibromuscular tissue traversed by the vascular, lymphatic and nerve supplies to the cervix.

Concept of Squamocolumnar Junction

- During childhood and perimenarche the squamocolumnar junction is very close to the external os.
- After puberty and during the reproductive period, eversion of the columnar epithelium onto the ectocervix takes place. The squamocolumnar junction is in the ectocervix, far away from the external os.

- The change in location is due to the exposure of everted columnar epithelium to the acidic vaginal environment.
- The buffer action of the mucus covering the columnar cells is interfered with, leading to destruction and eventual replacement of the columnar epithelium by the newly formed metaplastic squamous epithelium.

SCREENING FOR CERVICAL CANCER

- The guidelines of the American Cancer Society (ACS) are as follows:
  - All women should begin cervical cancer screening at age 21.
  - Women 21 to 29 years should have a Pap test every 3 years. They should not be tested for HPV unless it is needed after an abnormal Pap test result.
  - Women 30 to 65 years should have both a Pap test and an HPV test every 5 years/ Pap test alone every 3 years
  - Women > 65 years who have had regular screenings with normal results should not be screened and those who have been diagnosed with cervical pre-cancer should continue to be screened.

SCREENING METHODS

i. Clinical methods.
ii. Cellular and molecular methods.

Clinical Methods

i. **Pelvic examination**
   - It is the first step in diagnosing cervical cancer.
   - This includes per speculum inspection to look for any abnormalities like erosion, ulcer, or a mass.

ii. **VIA /Visual inspection using acetic acid**
   - A simple visual screening test using vinegar (acetic acid) has the potential to dramatically reduce the number of deaths from cervical cancer in the developing world.
   - This is according to findings from a large, randomized study of 150,000 women in India, where the use of the screening tool yielded a 31% reduction in cervical cancer mortality.
   - *Principle of test:* Application of 5% acetic acid leads to reversible coagulation, or precipitation of the cellular proteins.
   - *Interpretation of VIA*
   - *CIN/invasive cancer:* Acetowhite areas that are dense, thick and opaque with well-demarcated margins and delayed reversal.
   - *Squamous metaplasia, leukoplakia, condyloma:* Acetowhite areas which are pale, thin, translucent, and patchy with ill-defined margins and immediate reversal.

iii. **VILI/Visual inspection with Lugol's iodine**
   - *Principle of the test:* The glycogen present within the cells binds with iodine to give a brown color.
   - The ectocervical squamous epithelium and the endocervical squamous metaplastic epithelium are glycogenated, while the columnar epithelium does not contain glycogen.

- Areas of CIN and invasive cancer do not take up iodine (as they lack glycogen).
- Areas with leukoplakia (hyperkeratosis) do not stain with iodine.
- Condyloma may not, or occasionally may only partially stain with iodine.
- In low resource settings, screening of carcinoma cervix by Pap smear can be replaced by cheaper and easily available visual methods like VILI, which has the high sensitivity to detect any grade of dysplasia, with a reasonable specificity.
- Even when screening with Pap smear is available, it should be combined with visual screening methods like VILI.
- This is because many cases of CIN missed by Pap smear are picked up by the visual tests and combined testing reduces the number of biopsies taken based on either test alone.

iv. **Colposcopy**

- A colposcope is a low-power, binocular field microscope with a powerful light source used for magnified visual examination of the uterine cervix to help in the diagnosis of cervical neoplasia.
- Following are the indications of colposcopy:
  - Suspicious looking cervix
  - CIN 1/2/3 on cytology
  - Positive on VIA
  - Positive on VILI
  - Persistent CIN 1 features after 18 months
  - Infection with HPV
  - Persistent unsatisfactory smear.
- **Modified Reid colposcopic index**
  - Use of a scoring or grading system may guide colposcopic interpretation and diagnosis in a less subjective manner and helps to develop a systematic approach to colposcopy.
  - The modified Reid colposcopic score (Table 12.1) based on the colposcopic index proposed by Reid and Scalzi (1985) is quite useful for this purpose.
- **Scoring**
  - A score of 0 to 2 points = Likely to be CIN 1
  - 3–4 points = Overlapping lesion: Likely to be CIN 1–2
  - 5 to 8 points = Likely to be CIN 2–3 lesions

v. **Cervicography**

- Cervicograms are replicate photographs of the cervix taken after the application of 5% acetic acid, using a camera with a fixed focal length and internal light source.
- The images are projected onto a screen at a fixed distance to simulate magnification and are interpreted by a trained evaluator.
- The major limitation is poor sensitivity among older women, whose transformation zones are often beyond the field of vision.
- In later large-scale evaluations, cervicography proved insufficiently accurate to serve as a stand-alone screening test.

vi. **Spectroscopy**

- **Principle of fluorescence:** The basic principle is to irradiate tissue samples with CW laser and detect the native fluorescence from fluorophores such as proteins, amino acids, and co-enzymes present in the tissues, *in vitro* and *in vivo*.
- The fluorescence spectra provide crucial information on biochemical as well as morphological aspects of the tissue.

Table 12.1: Modified Reid colposcopic index

Colposcopic signs	*Zero point*	*One point*	*Two points*
Color	Low-intensity acetowhitening (not completely opaque); indistinct acetowhitening; transparent or translucent acetowhitening; Aceto-whitening beyond the margin of the transformation zone; Pure snow-white color with intense surface shine	Intermediate shade - gray/white color and shiny surface (most lesions should be scored in this category)	Dull, opaque, oyster white; gray
Lesion margin and surface configuration	Microcondylomatous or micropapillary contour[1] Flat lesions with indistinct margins; Feathered or finely scalloped margins; Angular, jagged lesions; Satellite lesions beyond the margin of the transformation zone	Regular-shaped, symmetrical lesions with smooth, straight outlines	Rolled, peeling edges; Internal demarcations between areas of differing colposcopic appearance—a central area of high-grade change and peripheral area of low-grade change
Vessels	Fine/uniform-calib vessels-closely and uniformly placed Poorly formed patterns of fine punctation and/or mosaic; Vessels beyond the margin of the transformation zone; Fine vessels within microcondylomatous or micropapillary lesions	Absent vessels	Well-defined coarse punctation or mosaic, sharply demarcated—and randomly and widely placed
Iodine staining	Positive iodine uptake giving mahogany-brown color; Negative uptake of insignificant lesion, i.e., yellow staining by a lesion scoring three points or less on the first three criteria Areas beyond the margin of the transformation zone, conspicuous on colposcopy, evident as iodine-negative areas (such areas are frequently due to parakeratosis)	Partial iodine uptake—variegated, speckled appearance	Negative iodine uptake of significant lesion, i.e. yellow staining by a lesion already scoring four points or more on the first three criteria

- Any change in the concentrations, quantum efficiency, or binding sites gets reflected in their fluorescence properties, giving rise to a contrast in fluorescence between the normal and the cancerous tissue sites.
- The dominant fluorophores found in the cervical tissue are collagen (peak around 400 nm) and NADH (peak around 460 nm).

Cellular and Molecular Methods

i. **Conventional exfoliative cervicovaginal cytology, i.e. the cervical (Pap) smear**

- The Papanicolaou test (Pap smear) is a method of cervical screening used to detect potentially precancerous and cancerous processes in the cervix.
- The test was independently invented by Dr. Georgios Papanicolaou and Dr. Aurel Babeș and named after Papanicolaou.
- After inserting a speculum into the vagina, the health care provider then collects a sample of cells from the outer opening or os of the cervix by scraping it with an Ayre spatula.
- An endocervical brush or a plastic-fronded broom is rotated in the central opening of the cervix.
- The cells are placed on a glass slide and taken to the laboratory to be checked for abnormalities.
- The sample is stained using the Papanicolaou technique.
- An optimal cervical specimen includes sampling of the squamous epithelium (Ectocervix) and columnar epithelium (Endocervix) and in particular the transformation zone.
- Spread the material collected on the spatula/cervix brush evenly over the slide with painting action and single smooth stroke motion using both sides.

ii. **Liquid-based cytology**

- It means cytology (study of cells) through a liquid medium.
- Cells are collected and the placed directly into a liquid preservative, rather than transferred to the slide.
- It removes some of the mucus, bacteria, yeast, and pus cells in a sample and can spread the cervical cells more evenly on the slide.
- FDA has approved ThinPrep and SurePath processors for liquid-based cytology.
- In ThinPrep, filtration and collection of vacuum-packed cells on a membrane which are transferred to the glass slide.
- In SurePath, centrifugation and sedimentation through a density gradient.
- Other processors which are not FDA approved include Cytoscreen, Turbitec, Cellslide, Papspin, Centrifugation and sedimentation by manual techniques, CellPrep Plus LBC Processor II, and Automatic (Capping, Cell transfer, Filter supply).

SurePath

It is based on centrifugation and sedimentation through a density gradient.

i. **Cell collection method**

- The sample is taken using a broom-like device (e.g. Rovers Cervex-Brush). Then, the head of the collection device is 'popped' or removed into the cytology collection vial containing the preservative provided by SurePath.
- The vial is then securely capped, labeled and sent to the laboratory for processing.
- The preservative contains 24% ethanol and is stable for 36 months from the date of manufacture at room temperature.
- The samples are stable for 4 weeks at room temperature and for 6 months if refrigerated.

ii. **Sample processing**
- A homogenous monolayered smear of cells is obtained via SurePath.

iii. **ThinPrep**
- This is based on filtration and collection of vacuum-packed cells on a membrane and transferring them to glass slide in a ThinPrep processor.
- A comparison between conventional cytology and liquid-based cytology is shown in Table 12.2.
- The advantages and disadvantages are shown in Table 12.3.
- To summarize, LBC smears are characterized by:
  - Clean background
  - Cells are more circular.
  - Cytoplasmic and nuclear features are more easily identified and
  - Cells with low-grade dyskaryosis, especially koilocytosis can be easily identified.

Table 12.2: Conventional cytology versus liquid-based cytology

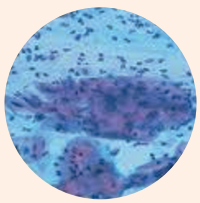
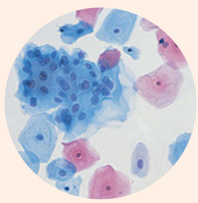
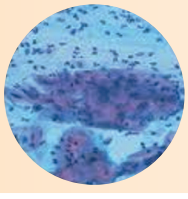
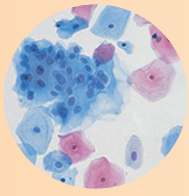
Technique	*Conventional*	*Liquid based*
		
Sampling device	Wooden spatula, cytobrush	Plastic spatula, cytobrush, cytobroom
Sample handling	Making a smear on the glass slide	Rinse or detach and place collection device in a vial of preservative fluid
Fixation	Manual spray fixation	Immediate and homogeneous fixation in methanol/ethanol
Lab preparation	Ready for staining	Semi-automated instrument to prepare slides before staining
Screening	Uneven distribution of cellular materials, 300,000 cells to screen	Need morphology re-training, uniform distribution of cells, fewer (75,000) cells to screen

Table 12.3: Advantages and disadvantages of conventional cytology *versus* liquid-based cytology

Technique	*Advantages*	*Disadvantages*
Conventional Pap test 	⮝ The gold standard for 50 years ⮝ Comfortable with the method ⮝ Ready for staining when arrives in lab ⮝ Inexpensive	⮝ >80% of materials lost ⮝ More cells to screen ⮝ Uneven distribution of cells, fixation artifacts

Contd.

Table 12.3: Advantages and disadvantages of conventional cytology *versus* liquid-based cytology

Technique	*Advantages*	*Disadvantages*
Liquid-based cytology 	⅄ Minimal loss of collected material ⅄ Ease of use for smear taken ⅄ Limited obscuring material—fewer unsatisfactory slides ⅄ Even distribution of cells, cleaner backgrounds ⅄ Fewer but well-preserved cells to examine—reduction of screening time ⅄ Residual materials for additional studies ⅄ Enable automated screening	⅄ Training required ⅄ Increased workload in prep lab ⅄ Increased costs

- According to a study conducted in 2010 by the American Cancer Society, excessive mucus remains a potentially limiting factor in the ThinPrep Pap System.
- This is in contrast with the SurePath system which manages mucus by a cell enrichment process that results in undiminished cell recovery as observed in this study's test conditions.
- Despite several advantages of liquid-based cytology over conventional cytology, Ronco G. et al conducted a randomized controlled trial with >45,000 samples found that the liquid-based cytology is not statistically significantly different from the conventional cytology in terms of sensitivity for detection of CIN-II.
- Similarly work by Arbyn M. et al concluded that liquid-based cytology is neither more sensitive nor more specific for the detection of high-grade CIN compared with the conventional Pap test.

iii. Automated Cervical Screening Techniques

- These techniques use an automated microscope that captures images with high speed image analysis followed by the use of computer algorithms to identify and/or evaluate abnormal cells.
- It took more than 40 years before the first successful commercial systems appeared.
- And still automated screening is not sufficiently cost-effective to completely replace the visual screening.

First Generation Systems

- The Cytoanalyzer project in the US was the first attempt at building an automated screening device for Pap-smears.
- It generated two-dimensional histograms of nuclear size versus nuclear optical density.
- However it gave too many false alarms leading to rejection due to artifacts and the project failed in the early sixties because of this.

New Generation Systems

- When the first-generation systems were developed there were no interactive computers and no display units capable of showing digital images available.
- The parameters studied by the new generation systems are nuclear area, nuclear density, cytoplasmic area, N/C ratio and chromatin pattern.

- The USFDA-approved systems are as follows:
  - Papnet [Neuromedical Systems Inc (NSI)]
  - AutoPap (Neopath Inc, Redmond Washington, USA)
  - Tripath FocalPoint system.

Papnet

- It is composed of a scanner in a central processing facility and a review station in the cytology laboratory.
- It uses an artificial neural network, which is a type of artificial intelligence for pattern recognition and that helps to automate the search for abnormality and focus the review of suspicious cells by a trained cytologist.
- The scanner searches the conventionally prepared smear.
- Slide loaded on scanning light microscope by a robot arm.
- Examined at 3 magnifications:
  - 50X—material distribution on the slide
  - 200X, 400X—image processing algorithm
  - Identify abnormal cells.
- Storage of color images of these abnormal cells and clusters is done on a digital tape drive which are then reviewed by a cytotechnologist on a high-resolution color monitor.
- The results are interpreted by the cytotechnologist as
  1. Negative
  2. Requiring review of the glass slide.
- On review of the glass slide, the findings are interpreted as:
  1. Negative
  2. Abnormal and requiring pathologist review.
- The pathologist reviews the slide and determines the final diagnosis.

TriPath Focal Point

- Currently available automated cervical cytology screening device, for initial screening of cervical cytology slides.
- It identifies up to 25% of successfully processed slides as requiring no further review and at least 15% of all for a second manual review.
- It is intended to be used on both conventionally prepared and SurePath slides.
- Full-slide manual screening is required if one of the following conditions are met:
  1. Any potential abnormalities are identified in any of the field of views (FOVs).
  2. The instruments undergo a technical processing failure.
- Hence, while working manually, a cytotechnologist can analyze about 80 to 90 slides a day (regulations set the maximum at 100 per day).
- Using the FocalPoint, a human examiner can go through 170 slides per day.
- Following are the benefits of automation:
  - Guides the screeners to microscopic fields containing potentially abnormal cells
  - Improves interpretation
  - Reduces the labor required for manual screening
  - Provides a visual record of cells that have been identified as potentially abnormal

- The automation methods, however, are not devoid of technical challenges which are as follows:
  - *Specimen preparation:* The manual smearing and staining lead to uneven distribution, formation of clumps, overlapping cells and empty spaces.
  - *Scanning:* At a pixel size of 0.2 microns, a smear of 25 × 75 mm will give 31 billion pixels. Hence storage and transferring such huge data poses a big challenge. In contrast, in liquid-based preparation—the area of the sample is around 1/10 of that of a smear which is a great advantage when it comes to scanning.
  - *Segmentation of nuclei and cells:* Segmentation is to delineate each cell and/or cell nucleus, which is required to be done quickly, under different staining conditions and in the presence of disturbing object in the direct vicinity.
  - *Artifact rejection:* The automation methods must be able to analyze in the presence of RBCs, WBCs, bacilli and folded nuclei.

iv. HPV Testing

- Human papillomavirus (HPV) is a double-stranded DNA virus and is a member of the Papovaviridae family of viruses that is capable of infecting humans.
- Called papillomaviruses as certain types may cause warty growths or papillomas.
- More than 100 genetically distinct types of HPV have been identified—low-risk types and high-risk types as shown in Table 12.4.

Table 12.4: High-risk HPV and low-risk HPV

High-risk (oncogenic or cancer-associated) types	*Low-risk (non-oncogenic) types*
16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 82	6, 11, 40, 42, 43, 44, 54, 61, 72, 73, 81
HPV-16 is responsible for –60% of cervical cancer, HPV-18 for –10%, HPV-45 and HPV-31 for –4% each.	These cause benign or low-grade lesions and genital warts
HPV-33, HPV-52, and HPV-58 together account for –2% of cervical cancer	

- The risk factors are young age (<25 years), multiple sex partners, early age at first intercourse (16 years or younger) and male partner has (or has had) multiple sex partners.
- Structure of HPV: Double-stranded, circular DNA, approximately 7900 bp.
- Genes comprising early (E), and late (L) genes.
- The early genes regulate viral replication and some have transformation potential.
- In benign warts the HPV genome is maintained in a nonintegrated episomal form, while in cancers the HPV genome is integrated into the host genome.
- Integration of viral DNA is important for malignant transformation. However, integration site is random.
- Integration interrupts the viral DNA within the E1/E2 genes, leading to loss of the E2 viral repressor and overexpression of the oncoproteins E6 and E7.
- The oncogenic potential of HPV can be related to the products of two viral genes: **E6 and E7.**
- The **E7 protein** binds to the Rb protein, which displaces the E2F transcription factors (normally sequestered by RB), promoting progression through the cell cycle.
- E7 protein from high-risk HPV types has a higher affinity for Rb than does E7 from low-risk HPV types.

- The **E6 protein** has complementary effects.
- It binds to and mediates the degradation of p53 and BAX, a pro-apoptotic member of the BCL2 family and it activates telomerase.
- Like E7, E6 from high-risk HPV types has a higher affinity for p53 than E6 from low-risk HPV types.
- Thus, stimulation of expression of transcription factor **HIF-1**α occurs.
- Consequently, higher levels of **HIF-1**α expression in early-stage invasive cervical cancer are correlated to shorter overall survival time.

Significance of HIF-1α Expression in Cervical Cancer

- With normal p53, HIF-1α will be expressed only in hypoxic states. HIF-1***α*** binds and stabilizes p53 to induce apoptosis of hypoxic cells.
- However, in cells with integrated DNA of HPV, p53 is degraded. This leads HIF-1***α*** to stimulate neoangiogenesis via VEGF pathway for tumor cells, providing the vascularization necessary for cancer progression.
- Hence, anti-HIF-1α or anti-VEGF antibody treatment may control the progression of cervical cancer.

Molecular Testing of HPV

- HPV cannot be grown in conventional cell cultures.
- **Serological assays** have only limited accuracy as (antibodies remain detectable for many years), serology is not suitable for distinguishing present and past infections.
- Consequently, accurate diagnosis of HPV infection relies on the detection of viral **nucleic acid.**

Hybrid Capture 2

- It is the qualitative detection of Human Papillomavirus (HPV) and remains the current gold standard in HPV diagnostics.
- It is the only FDA-approved HPV DNA test commercially available for clinical use.
- **HC–1:** First introduced in 1995 by Digene Lab, used tube format.
- **HC–2:** Second generation test for HPV DNA, uses microtiter plate instead of tubes.
- **HC–3:** For research purposes.
- **Principle**
  - Based on the hybridization of the target HPV-DNA to labeled RNA probes in solution.
  - The resulting RNA–DNA hybrids are captured on microtiter wells.
  - These are detected by a specific monoclonal antibody and a chemiluminescent substrate providing a semi-quantitative measurement of HPV-DNA.
- **Preparation**
  - Before testing, do not douche or use tampons or vaginal medication for at least before 48 hours.
  - Best time to schedule test—8 to 12 days from the start of last menstrual period.
- **Handling of specimens**
  - Cervical swab samples may be held for up to 2 weeks at room temperature, 3 weeks at 4°C or 3 months at –20°C.
  - Testing should be performed within 3 weeks from the day the sample is collected.

- Two different probe cocktails are used:
  - One comprising probes for five low-risk genotypes 6, 11, 42, 43 and 44.
  - Other containing probes for 13 high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.
- **Run time:** 3 hours 30 minutes.
- **Limitations:**
  - It distinguishes between the high-risk and low-risk groups but does not permit the identification of specific HPV genotypes.
  - It is less sensitive than PCR.
- Overall, it is robust and reproducible as a screening assay.

Cervista HPV HR

- Cervista HPV HR is a qualitative, *in vitro* diagnostic test for the detection of DNA from 14 high-risk HPV types, namely types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.
- The Cervista HPV HR test uses the Invader chemistry, a signal amplification method for detection of specific nucleic acid sequences.
- This method uses two types of isothermal reactions: A primary reaction that occurs on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal.
- In the primary reaction, two types of sequence-specific oligonucleotides (i.e. a probe oligonucleotide and an Invader oligonucleotide) bind to the DNA target sequence.
- When these oligonucleotides overlap by at least one base pair on the target sequence, an invasive structure forms that acts as a substrate for the Cleavase enzyme.
- The enzyme cleaves the 5′ portion (flap) of the probe at the position of the overlap.
- A positive result indicates at least one of the 14 high-risk HPVs are present in the sample when fluorescent signal lies above the cut-off value.
  - Advantages include increased specificity, low to no cross-reactivity with low-risk HPV genotypes and lower required sample volumes (10 microliter).
- **Other techniques**
  - PCR
  - Amplicor HPV
  - Papillochek.

ROLE OF BIOMARKERS

- Dual staining by immunohistochemistry of Ki-67 and p16 is an indirect test.
- Hans Ikenberg et al investigated the p16/Ki-67 dual-stained cytology approach in a large prospective diagnostic screening study, the Primary ASC-US and LSIL Marker Study (PALMS).
- This pan- European study was performed to estimate the sensitivity and specificity of the p16/Ki-67 dual-stained cytology and to compare it with Pap cytology testing in a routine European cervical cancer screening population of women 18 years or older, and to HPV testing in women 30 years or older.
- They concluded that the p16/Ki-67 dual-stained cytology combines superior sensitivity and non-inferior specificity over Pap cytology for detecting CIN2+.
- It suggests a potential role of dual-stained cytology in screening, especially in younger women where HPV testing has its limitations.

- **Prevention of HPV can be pursued by:**
  - Following safe sexual practices
  - Using condoms
  - Routine Pap testing and screening for cervical cancer
  - Vaccination is now available.
- **Clinical goals against HPV includes:**
  - Prevention of genital warts
  - Prevention of cervical dysplasia
  - Immunotherapy of genital warts
  - Prevention of Symptomatic anogenital disease
  - Immunotherapy of cervical cancer
  - Prevention of other cancers (oral, esophageal, head and neck) caused by these HPVs.
- **Vaccine**
  - HPV L1 is a major capsid protein of the virus which is the antigen used for immunization.
  - L1 protein expressed in yeast cells using recombinant technology.
  - L1 proteins self-assemble into virus-like particles (VLP).
  - VLPs are noninfectious and nononcogenic.
- A comparison of available HPV vaccines is shown in Table 12.5.

Table 12.5: Gardasil versus Cervarix vaccine

Vaccine	*Gardasil*	*Cervarix*
Company	SP MSP Merck	GSK
Vaccine Type	L1 VLP(20/40 mg)	L1 VLP (20/20 mg)
Produced in	Yeast	SF-9 insect cells
Disease	Genital warts, cervical dysplasia and cancer	Cervical dysplasia and cancer
Target HPVs	HPV 6, 11, 16 and 18 (Quadrivalent)	HPV 16 and 18 (Bivalent)
Injection schedule	0.5 mL intramuscular 0, 2 and 6 months	0.5 mL intramuscular 0, 1 and 6 months
Adjuvant used	Aluminum hydroxyphosphate sulphate	Aluminum hydroxy-phosphate sulphate
Safety	Well tolerated, minor adverse effects, no vaccine-related SAE	same
Efficacy in preventing 100% persistent infection	89%	100%
Efficacy in preventing 100% CIN lesion	100%	100%
Cross protection	Not Known	HPV 45 and 31

- US FDA has approved the administration of HPV vaccine at ages 9 to 26 years.
- Side effects—nausea, vomiting, dizziness and pain at the injection site.
- Should not be used in those patients who are allergic to the constituents.
- Cost per dose is Rs 2200 per dose for Cervarix and Rs 3000 per dose for Gardasil.
- The vaccine is stored at 2°C to 8°C and is to be protected from light.

Vaccination in Males

- In males, Gardasil may reduce the risk of genital warts and precancerous lesions caused by HPV.
- The vaccine has been FDA-approved for use in males age 9 to 26 for the prevention of genital warts and anal cancer.

POINTS TO REMEMBER

- Cervical cancer is the leading cancer among Indian females.
- The screening methods include clinical, cellular and molecular methods.
- VIA and VILI are very useful methods in resource-limited settings.
- Conventional cytology and liquid-based cytology are both routinely available methods.
- They both have their relative advantages and disadvantages.
- Automated screening techniques have not proven to be cost-effective approach.
- Out of the various techniques available for HPV detection, Hybrid capture is the only US FDA-approved technique.

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13 Advances in Understanding of Cutaneous Mesenchymal Neoplasms

Harsh Batra

INTRODUCTION

- Cutaneous mesenchymal tumors arise from supporting mesenchymal tissues of dermis and subcutis (fibrous connective tissue, blood vessels, nerves, adipose tissue and smooth muscle).
- They often pose significant diagnostic challenges.
- Many such entities are rare and show clinical and histologic overlap with other mesenchymal and non-mesenchymal lesions.
- In recent years, there have been several important refinements in the classification of cutaneous mesenchymal neoplasms.
- These include the description of new tumor types, along with the identification of novel and recurrent molecular genetic findings.
- These changes have provided new insights into tumor biology and significant clinical consequences with regard to diagnostics, management and prognostification.
- Newly described entities include:
  a. Pseudomyogenic hemangioendothelioma,
  b. Hemosiderotic fibrolipomatous tumor
  c. Fibroblastic connective tissue nevus

Newly Described Cutaneous Mesenchymal Neoplasms

Pseudomyogenic Hemangioendothelioma

- Pseudomyogenic hemangioendothelioma, which has also been referred to as epithelioid sarcoma-like hemangioendothelioma is a rare soft tissue tumor of intermediate biological potential.
- This clinically and histologically distinctive tumor typically arises in young adults, with a peak incidence at age 30 years and a marked (4:1) male predilection.
- Patients usually present with dermal or subcutaneous nodules on the extremities, most commonly the lower limbs, and 66% of patients have multifocal disease at presentation.
- Tumors are [18F] fluorodeoxyglucose-avid on positron emission tomography scan, which helps to delineate the extent and multifocality of the disease.
- 50% of the patients can show the development of additional nodules in the same region, however, metastasis is rare.

- **Microscopy:**
  - Tumor has infiltrative edges.
  - Sheets and lose fascicles of spindle cells with abundant brightly eosinophilic cytoplasm, vesicular nuclei, small nucleoli.
  - Cells are cytologically bland with mild atypia and low mitotic activity.
  - Focal areas of epithelioid morphology can be seen.
  - 50% have prominent neutrophilic infiltrate.
  - Some tumor cells have rhabdomyoblast like appearance.
- **Immunohistochemistry:**
  - Broad spectrum cytokeratins (CK AE1/AE3 or PAN-CK or MNF-116)
  - Nuclear expression of ERG and FLI-1 (endothelial differentiation)
  - Retained INI-1 (SMARCB1)- differentiates from epithelioid sarcoma which shows loss of INI-1 expression.
  - Variable expression of CD31, SMA, CAM5.2, EMA.
  - The tumor is negative for CD34, S-100 and Desmin.
- **Differential diagnosis**
  - *Epithelioid sarcoma:* It lacks plump myoid appearing spindle cells morphology and fascicular and sheet-like growth arrangement. Loss of INI-1 expression and positive staining for EMA, CD34 and negative staining for ERG or FLI-1.
  - *Epithelioid hemangioendothelioma:* It shows the presence of intracytoplasmic vacuoles and corded and nested patterns of growth. Strong keratin expression is rare.
  - *Spindle cell SCC:* It is seen in sun-damaged skin of elderly patients and shows more cytological atypia. Expression of FLI-1 and CD-31 rules out SCC.
  - *Cellular benign fibrous histiocytoma:* It lacks plump cytomorphology, intense cytoplasmic eosinophilia and neutrophilic infiltrate. There is no CK expression.
  - *Myofibroblastic or smooth muscle neoplasms:* Strong and diffuse keratin expression, at most focal SMA and negative Desmin staining rules out the possibilities of these two lesions.
- **Genetics and treatment**
  - Recurrent balanced translocation t(7;19) (q22;q13) results in fusion of SERPINE1-FOSB which is pathognomonic for PHE.
  - Conservative management is the treatment of choice often in the form of complete but narrow excision or curettage.

Hemosiderotic Fibrolipomatous Tumor

- Hemosiderotic fibrolipomatous tumor is an uncommon, locally aggressive neoplasm of uncertain lineage.
- It arises almost invariably as a slow-growing mass in the ankle or foot region of women during the fifth and sixth decades.
- It was previously thought to be a reactive process associated with trauma.
- It has a high rate of local recurrence.
- It has overlapping molecular features with two other soft tissue neoplasms—myxoinflammatory fibroblastic sarcoma (MIFS) and pleomorphic hyalinizing angiectatic tumor (PHAT).
- **Gross and microscopy:**
  - It grossly ranges in size from 1 to 10 cm.
  - Cut surface shows dark-yellow or lipoma-like appearance with intervening white nodules and septa.

- These are unencapsulated and may have well-circumscribed or poorly-defined cell borders.
- **Microscopically,** HFLT is composed of two cell types—adipocytes and bland spindle cells.
- A variable amount of mature adipocytes present, within which nodules and septa of short plump spindle cells arranged in storiform or fascicular pattern.
- The spindle cells often wrap around individual adipocytes in a honeycomb pattern reminiscent of DFSP (dermatofibrosarcoma protuberans).
- The spindle cells have vesicular nuclei, indistinct nucleoli, moderate cytoplasm and indistinct cell border.
- Prominent golden-brown hemosiderin pigment is seen within macrophages, within the spindle cell themselves and in the adjacent stroma.
- Spindle cell components may also contain osteoclast-like giant cells, mixed chronic inflammatory infiltrate, entrapped blood vessels and nerves.

- **Relation with MIFS and PHAT**
  - Myxoinflammatory fibroblastic sarcoma (MIFS) is a relatively hypocellular neoplasm that shows prominent inflammatory cell infiltrate, myxoid stroma and admixed atypical cells with "RS"-like nuclei.
  - Prominent myxoid areas seen in MIFS can be seen in HFLT and "hybrid" tumors showing features of both HFLT and MIFS have been described.
  - Recently same chromosomal translocation t(1:10) (p22:q24) and fusion gene TGFBR3-MGEA5 have been identified in both neoplasms.
  - The same fusion gene has also been identified in PHAT, a rare locally aggressive mesenchymal tumor that may show striking histological overlap with HFLT and MIFS.
  - PHAT shows distinctive vessels with hyalinization and fibrinoid change within their walls and an abundance of pleomorphic cells without discernible mitotic activity.
  - MIFS show metastasis.
- **Differential diagnosis:**
  - *Plexiform fibrohistiocytic tumor:* PFHT is composed of a more polymorphous cellular population, comprising frequent giant cells, spindled fibroblast cells and histiocytoid cells within small, distinct nodules. The adipocytic component is less prominent than in HFLT, and, unlike in HFLT, the tumor cells are usually positive for SMA.
  - *Dermatofibrosarcoma protuberans:* Honeycomb pattern of infiltration of adipose tissue may be reminiscent of HFLT but DFSP typically and is more cellular, does not contain hemosiderin pigment.
  - *Spindle cell lipoma:* Heterogeneous-sized adipocytes and myxoid changes are typical of spindle cell lipoma. Hemosiderin pigment is absent.
  - *Well-differentiated liposarcoma:* Lipoblasts and scattered pleomorphic stromal cells are characteristics of well-differentiated liposarcoma.
- Immunohistochemistry is generally unhelpful with just the spindle cells being CD34 positive
- **Genetics:** TGFBR3-MGEA5 fusion gene is seen.
- **Treatment:** These tumors have a high propensity for local recurrence and they are included in the intermediate category. Complete excision is generally curative.

Fibroblastic Connective Tissue Nevus

- Connective tissue nevi are benign hamartomatous lesions of papillary or reticular dermis.
- These occur in isolation or are associated with Proteus and Cowden syndrome
- Solitary, slow-growing, painless cutaneous plaque or nodule, typically on the head and neck or trunk of young girls (average age 10 years).
- It is small in size, ill-defined and range from 3–20 mm.
- **Microscopy:**
  - Proliferation of bland, spindle fibroblastic/myofibroblastic cells with pale eosinophilic cytoplasm arranged in a disorderly fashion in the reticular dermis with extension in the superficial subcutis.
  - Intersecting bundles of spindle cells characteristically ramify through the dermis and entrap appendages.
  - Adipocytes are typically found in the abnormally superficial location and the overlying epidermis often shows papillomatosis.
  - *IHC:* The most consistent immunohistochemical finding is CD34 expression, which is found in 87% of cases and typically shows a weak and multifocal staining pattern 21; S100 and desmin are usually negative.
- **Differential diagnosis:**
  - *Plaque stage DFSP:* Uniform proliferation of mild atypical fibroblasts arranged in a storiform pattern. However, in some cases, the storiform pattern may be inconspicuous. In such instances absence of epidermal papillomatous hyperplasia, presence of a hypocellular zone just beneath the epidermis, careful assessment of uniform but atypical nuclei and strong, diffuse CD34 positivity in lesional cells favor the diagnosis of DFSP.
  - *Dermatofibroma:* Clinically presents in shoulder and arm of young adults (median age 30 years) in contrast to younger age presentation of FCTN. Fascicles of tumor cells are typically oriented, parallel to the overlying epidermis. It appears less cellular than FCTN. Lesional cells of dermatofibroma appear more eosinophilic. CD34 positivity focally (<25% cases in contrast to >87% cases of FCTN) and SMA positivity (focal in FCTN).
  - *Pilar leiomyoma:* Often painful. Ill-defined bundles of well-differentiated smooth muscle cells in the reticular dermis associated with epidermal hyperplasia. Pilar leiomyoma has a more brightly eosinophilic cytoplasm. It is consistently desmin positive.
- **Treatment:**
  - Complete excision is the treatment.
  - It is an entirely benign lesion.
  - Likelihood of recurrence is very low even in the setting of incomplete excision.

RECENT GENETICALLY CHARACTERIZED CUTANEOUS MESENCHYMAL NEOPLASMS

Conventional Fibrohistiocytoma (FH)

- Also called dermatofibroma, it is the most common cutaneous mesenchymal tumor.
- It has many histological variants, viz. cellular, atypical, aneurysmal, lipidized and epithelioid.
- Cellular and atypical type has a higher rate of recurrence.

- Exceptionally rare cases of metastasis of morphologically benign FH occur, and these cases are more likely to be histological variants of FH such as cellular, aneurysmal, or atypical types, than conventional FH.
- Conventional or usual-type FH typically presents as a solitary reddish-brown nodule on the extremities, particularly in young to middle-aged women.
- These lesions are usually small, the majority measuring <10 mm.
- **Genetics:**
  - The biology of FH has been a matter of debate, whether neoplastic or reactive.
  - Biological evidence has confirmed that it is a true neoplasm.
  - In 2014, gene fusions involving a protein kinase C (PKC) gene in cases of benign FH were investigated at the RNA level.
  - PKCs are serine-threonine kinases that are involved in a variety of cellular processes and are implicated in tumor development.
  - The PKC isoforms PRKCB and PRKCD have been detected fused with genes encoding membrane-associated proteins (PDPN, CD163, and LAMTOR1).
  - These gene fusions are thought to result in a chimeric protein that causes constitutive activity of PKC and promotes tumorigenesis.

Epithelioid Fibrous Histiocytoma (EFH)

- A variant of benign cutaneous FH with prominent epithelioid morphology.
- Recent data shows that EFH has a distinct immunohistochemical and genetic profile from conventional FH being ALK-translocated neoplasm.
- According to the current definition, it is a variant of FH with at least 50% of lesional cells having epithelioid morphology.
- It presents as a solitary dermal-based nodule most commonly in the extremities of young-to-middle-aged adults.
- **Microscopy:**
  - Well-circumscribed and exophytic, with an associated epidermal collarette, features that are uncommon in conventional FH thus helping in differentiation.
  - Consists of monotonous, plump epithelioid cells with abundant pale eosinophilic cytoplasm, vesicular nuclei and small nucleoli.
  - Binucleate cells are present in nearly all cases but in contrast to conventional FH, multinucleate giant cells are less common. Lateral entrapment of collagen is usually absent.
  - Tumors often contain small-to-medium-sized vessels with perivascular accentuation of tumor cells.
  - Mitotic activity is low.
  - Follows a benign course with only rare reports of local recurrence.
  - Negative for SMA and desmin in contrast to classic FH. EMA expression that is absent in classic FH, is present in 64% cases of EFH. It retains the expression of INI-1.
- **Genetics:**
  - ALK rearrangements resulting in the formation of VCL–ALK and SQSTM1–ALK fusions were originally described in two cases of 'atypical' FH with epithelioid features.
  - A larger, more recent series of 33 epithelioid FHs showed diffuse cytoplasmic expression of ALK in 88% of cases with corresponding gene rearrangement being

identified in IHC-positive cases examined by fluorescence *in situ* hybridization (FISH).
- CD30 expression was found in 62% of cases in the same series.

- **Differential diagnosis**
  - *Cutaneous syncytial myoepithelioma:* It often causes the most diagnostic confusion, owing to the shared expression of EMA and somewhat overlapping morphological features, but lacks ALK expression.
  - *Epithelioid sarcoma:* It can have a similar clinical presentation and cytomorphology to EFH (but usually found deeper and shows more cytological atypia) and shares EMA expression, but can be distinguished from EFH by immunopositivity for cytokeratins and CD34, and loss of nuclear expression of INI1.
  - *Spitz nevi:* Spindle and epithelioid cells arranged in discrete nests. Epidermis is often hyperplastic, elongated rete ridges and Kamino bodies. Although 10% spitz nevi show ALK re-arrangement, expression of S-100, Melan-A and absence of EMA expression rules out EFH.

Cutaneous Myoepithelial Tumors

- Cutaneous myoepithelial tumors constitute a relatively recent addition to the myoepithelial tumor family, and are histologically similar to their salivary gland counterparts.
- Myoepithelial tumors of the skin comprise three main subtypes: Chondroid syringoma (or 'mixed tumor'), cutaneous myoepithelioma, and myoepithelial carcinoma.
- **Mixed tumor,** or chondroid syringoma as it is known in the skin, includes myoepithelial neoplasms that show tubuloductular differentiation and are analogous to pleomorphic adenoma of the salivary gland, both morphologically and genetically.
- Mixed tumors show rearrangement of the pleomorphic adenoma gene 1 (PLAG1), located on chromosome 8q12. IHC for PLAG1 is useful to confirm this diagnosis. These tumors are benign and although local recurrence may occur in up to 20% of cases.
- Cutaneous myoepithelioma is defined by the presence of a pure myoepithelial cell population without tubuloductal differentiation.
- Occurs in patients of all ages, with a 3:1 male predominance.
- Patients present with a long-standing, painless dermal-based mass, most commonly on the extremities and with an average size of 10 mm.
- **Microscopy:**
  - These neoplasms show the spectrum of morphology with the majority having nodular or lobulated architecture and is composed of epithelioid, plasmacytoid or spindle cells set in a myxoid or hyalinized matrix.
  - In 2013, this subtype of 'cutaneous syncytial myoepithelioma' was characterized in a larger series. This distinct subset lacks any significant matrix, and instead shows a solid, well-circumscribed proliferation of ovoid, spindled or histiocytoid cells with pale eosinophilic syncytial cytoplasm, bland nuclei, and minimal mitotic activity.
- **IHC**
  - These groups of tumors co-express keratin and/or EMA, S-100, GFAP with variable staining for p63, SMA, calponin.
  - Cutaneous syncytial myoepitheliomas show characteristic co-expression of S100

and EMA with a weak and multifocal expression of GFAP in 75% of cases and in contrast to most myoepithelial tumors, keratin expression is usually absent.
  - EWSR1 re-arrangement has been detected in 29–44% cases of cutaneous myoepithelioma and 82% cases of syncytial variant.
- **Differential diagnosis:**
  - *Epithelioid fibrous histiocytoma:* Sometimes causes morphologic similarities. EFH is usually S-100 negative and ALK-positive.
  - *Early-stage juvenile xanthogranuloma:* Expresses CD-68 and CD-163 and does not express myoepithelial markers.

Cutaneous Vascular Tumors

Epithelioid Hemangioendothelioma

- It is a rare, malignant tumor that shows endothelial differentiation and can occur in a variety of anatomical sites, including soft tissue, bone, lung, liver, and skin.
- It develops as a solitary painful mass in superficial or deep soft tissue or it occurs multifocally in a localized region.
- Those tumors that arise from vessels usually have a variegated, white-red color and superficially resemble organizing thrombi.
- Microscopy shows epithelioid endothelial cells with intracytoplasmic lumina and mild cytological atypia.
- **Genetics:**
  - It is recently genetically characterized and found to be a 'translocation-associated sarcoma'.
  - In 2001, a t(1;3) (p36;q25) translocation was identified in two cases of epithelioid hemangioendothelioma.
  - In 2011, the genes involved in the translocation, WWTR1 on 3q25 and CAMTA1 on 1p36, were cloned.
  - WWTR1 is a gene involved in transcriptional factor activation and signaling in the Hippo pathway that is normally expressed in endothelial cells
  - CAMTA1 belongs to a family of calmodulin-binding transcription activators, and has been implicated as a tumor suppressor gene that is frequently lost in neuroblastoma and gliomas.
  - WWTR1–CAMTA1 fusion gene has been identified in ~90% of EHE cases with classic morphology and is not found in morphological mimics.
  - A subset of EHEs negative for the WWTR1–CAMTA1 fusion gene were shown to harbor a YAP1–TFE3 fusion gene.
  - This latter subtype was found mainly in soft tissues of young adults, and shows distinct well-formed vasoformative channels and brightly eosinophilic cytoplasm
  - Nuclear expression of CAMTA1 has very recently been shown to be a sensitive and specific surrogate marker for the fusion gene, and expression is seen in >90% of EHEs.
  - Expression of CAMTA1 is not seen in epithelioid AS, other epithelioid vascular tumors or soft tissue tumors of other types. This application is particularly useful for distinguishing those EHEs with marked cytological or architectural atypia ('malignant' EHEs) from AS.
  - Of those EHEs negative for CAMTA1, the vast majority will show expression of TFE3, but TFE3 is a less specific surrogate marker for the YAP1–TFE3 gene

fusion—in a recent study, only positive cases with a strong, diffuse staining pattern correlated with the presence of the fusion gene.

RADIATION-ASSOCIATED VASCULAR LESIONS

- Radiation-associated angiosarcoma was historically the least common subtype of cutaneous angiosarcoma.
- However, the incidence has increased in recent years, owing to the combination of conservative excision with radiation therapy now being the standard of care in early-stage breast cancers.
- The development of secondary cutaneous AS typically occurs after a latency period of several years.
- Angiosarcoma arising secondary to radiation for breast cancer usually occurs within the dermis and subcutaneous tissue.
- Secondary AS of the skin is associated with an aggressive clinical course, characterized by frequent local recurrences and early metastasis.
- Although radiation-associated AS is relatively rare, atypical radiation-associated vascular lesions/proliferations (AVLs) occur more commonly in radiated skin.
- **Atypical radiation-associated vascular lesions:**
  - AVLs present clinically as small papules, usually <5 mm, with red to brown discoloration.
  - Histologically, AVLs consist of scattered small lymphatic-like vascular spaces in the dermis that are often dilated or irregular in shape and lined by hyperchromatic endothelial cells.
  - In contrast to cutaneous AS, AVLs lack complex anastomosing or dissecting architecture, endothelial multi-layering, marked cytological atypia, and mitotic activity.
- **Genetics:** Some cases of AVL may be extremely difficult to distinguish from well-differentiated vasoformative AS morphologically, but recent studies have identified MYC amplification and MYC protein overexpression as a consistent finding in secondary AS, a finding that is not seen in AVLs, and is seen rarely in non-radiation-associated AS.
- IHC to identify MYC overexpression has become an extremely useful tool for distinguishing secondary/radiation-associated AS from florid AVLs.
- **Treatment:** The current treatment approach for radiation-associated AS is complete excision of all skin within the affected radiation field.
- In the case of breast cancer-associated radiation AS, this usually necessitates a large chest wall resection to include total mastectomy.
- For AVLs, because of the association with AS, complete excision (when feasible) and close clinical follow-up is warranted.

Epithelioid Hemangioma

- Epithelioid hemangioma (EH) is a rare benign vascular neoplasm composed of prominent epithelioid endothelial cells, often with cytoplasmic vacuoles, lining well-formed vascular lumina.
- EH arises at a wide variety of anatomical locations, including soft tissue, bone, penis, heart, colon, eye, and lymph nodes.

- The spectrum of EH ranges from so-called 'angiolymphoid hyperplasia with eosinophilia' (ALHE), in which a prominent lymphocytic and eosinophilic infiltrate is present, to cellular/solid and intravascular variants.
- **Genetics:**
  - In 2014 novel recurrent fusion gene ZFP36–FOSB was reported in a small subset of EHs showing increased cellularity, necrosis, and atypia
  - FOS rearrangements were found across different anatomical locations and histological variants; however, a higher prevalence was found in cellular EH and intraosseous lesions.
  - All cases of the ALHE variant examined lacked FOS rearrangement

REFINEMENTS IN CLASSIFICATION OF CUTANEOUS MESENCHYMAL NEOPLASMS

Intradermal Smooth Muscle Tumors

- 'Atypical intradermal smooth muscle neoplasm' (AISMN) represents a recent refinement in the classification of tumors formerly considered to be cutaneous leiomyosarcoma .
- These represent those atypical smooth muscle tumors that are confined to the dermis, with at most minimal extension into subcutaneous fat
- Historically, leiomyosarcomas of the dermis and subcutis were grouped together
- Recent studies have shown that primary dermal 'leiomyosarcomas' have an excellent prognosis as compared with their subcutaneous counterparts

Clinical Picture and Microscopy

- It presents in middle age to elderly adults, with M:F ratio being 4:1
- It presents as a solitary, slow growing cutaneous nodule on the trunk or lower limb generally <2 cm.
- It is a dermal based poorly circumscribed tumor
- Microscopy shows fascicles of tumor cells with bright eosinophilic cytoplasm, well-defined cell border and cigar-shaped nuclei, mild to moderate nuclear atypia and mitosis present.
- Extension into the superficial subcutis with a pushing border is very limited
- **Differential diagnosis:**
  - *Pilar leiomyoma:* Presents with multiple lesions. Lacks nuclear atypia and mitotic activity.
  - *Metastatic leiomyosarcoma:* It is differentiated with clinical correlation.
  - *Cellular benign fibrous histiocytoma:* These lack bright eosinophilic cytoplasm of AISMN and are less reminiscent of smooth muscle cells. Tumor cells are often positive for SMA, Desmin (focal) and negative for h-caldesmon. AISMN are positive for SMA, Desmin and h-caldesmon. Focal keratin positivity in 50% cases.

Pleomorphic Dermal Sarcoma/Unclassified Pleomorphic Sarcoma

- Formerly designated as cutaneous malignant fibrous histiocytoma, it is a, relatively uncommon dermal neoplasm.
- It shows some clinical and histological similarities to atypical fibroxanthoma, but that differs significantly with regard to clinical behavior.

- It is a rapidly growing, often ulcerated exophytic tumor on sun-damaged skin mostly on scalp of men in 7th–8th decade.

Microscopy

- Pleomorphic spindled and epithelioid cells with pale eosinophilic cytoplasm and marked nuclear atypia.
- Admixed giant cells are seen
- Mitotic count including atypical mitoses is high.
- Perineural invasion, lymphovascular invasion and necrosis may be seen.

IHC

- SMA positivity is seen in 70% cases.
- CD-31 positivity is seen in 48% cases (both tumor cells and intratumoral histiocytes)
- CD10 positivity is seen in 100% cases. But non-specific and of limited or no diagnostic utility.
- Pleomorphic dermal sarcoma often contains numerous intratumoral S-100 positive dendritic cells. Should not be confused with spindle cell melanoma.

Differential Diagnosis

- **Atypical fibroxanthoma:** It is a benign tumor with a low risk of local recurrence and no metastatic potential. Confined to the dermis whereas tumors with similar histological features to those of AFX but that show extension into the subcutaneous tissue are designated PDS and are associated with risks of local recurrence and distant metastasis of up to 30% and 20%, respectively.
- **Sarcomatoid SCC:** Pan-CK, CK-5 and p-63 helps to differentiate between these two entities.
- **Melanoma :** HMB-45, Melan-A, S-100, SOX-10 will be positive in melanoma.
- **AISMN or Leiomyosarcoma:** SMA, desmin and h-caldesmon are useful.
- Pleomorphic dermal sarcoma falls under the umbrella designation of 'undifferentiated /unclassified sarcoma', but with a better prognosis, and therefore not to be confused with, undifferentiated pleomorphic sarcoma of the subcutis or deep soft tissues.
- To date, no definite line of differentiation has been identified, PDS remains a diagnosis of exclusion, and requires evaluation with an appropriate immunohistochemical panel.

Genetics

- AFX and PDS remain incompletely characterized
- TERT promoter mutations are the most frequent mutations found in PDS to date. Recent data have shown that TERT mutations, with an associated ultraviolet (UV) signature very similar to those found in malignant melanoma, are present in 76% of PDS cases and 93% of AFX cases.
- The prognosis of PDS remains better than that of undifferentiated pleomorphic sarcomas of other body sites.
- Local recurrence rates, usually in the setting of incomplete excision, have been reported to be in the range of 20–28%.
- The incidence of metastatic disease now appears to be higher than initially suggested, with distant metastases occurring in 10% and 20% of cases in two recent studies.

POINTS TO REMEMBER

- Cutaneous mesenchymal neoplasms are a diagnostic challenge.
- Over recent years, refinements in tumor classification, the description of new tumor types and advances in the molecular understanding of a variety of different cutaneous mesenchymal neoplasms have occurred.
- This has had significant diagnostic, therapeutic and prognostic implications.
- Protein correlates of molecular alterations offer useful diagnostic adjuncts that are widely applicable in clinical practice.
- Examples are ALK and CAMTA1 expression for the diagnosis of epithelioid FH and EHE, respectively, and MYC overexpression in radiation-associated AS.
- Newly described entities widen the differential diagnostic considerations in certain settings.
- PHE was previously considered to be a variant of epithelioid sarcoma until it was recognized as a distinct clinicopathological and molecular entity.
- It has a penchant for local recurrence but not for metastasis.
- The refinement in the classification of PDS and atypical cutaneous smooth muscle neoplasms has significant clinical implications.
- Strict criteria now separate PDS from AFX, two entities that show similar clinicopathological features but striking differences in clinical behavior.

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Recent Advances in Identifying Cancer Cells in Stool Samples

14

Tanisha Singla

INTRODUCTION

- The term colorectal cancer refers to a slowly developing cancer that begins as a tumor or tissue growth (polyp) on the inner lining of the rectum or colon.
- Colorectal carcinoma is an important public health problem especially in developed countries.
- Worldwide, it is the 3rd most commonly diagnosed cancer which affects approx. 2.3 million patients each year.
- In the Indian scenario, it is the 8th most common cause of cancer. The current incidence in India is significantly lower than the west but there is an increasing trend due to changing dietary habits.
- There is a long natural history of about 10–15 years for development of colorectal cancer.
- High incidence and associated mortality, and the long natural history of colorectal carcinoma (CRC) makes it very suitable for population screening.
- It has been seen that removal of adenomas reduces the incidence of colorectal carcinoma by 76–90% (according to a national polyp study done in the USA).
- Screening tests for colorectal carcinomas can be divided into invasive and non-invasive tests. The invasive tests include the radiological workup like colonoscopy. Non-invasive tests include stool tests and blood tests.
- A two-step procedure is followed. First, a primary non-invasive investigation is done and if positive it is followed by colonoscopy.

STOOL TESTS

The fecal occult blood tests include:

1. Guaiac fecal occult blood test.
2. Fecal immunochemical test.

Guaiac Fecal Occult Blood Test

The stool guaiac test or guaiac fecal occult blood test (gFOBT) is one of the several methods that detects the presence of fecal occult blood (blood invisible in the feces).

Principle

The test involves placing a fecal sample on guaiac paper (containing a phenolic compound, alpha-guaiaconic acid) and applying hydrogen peroxide which, in the presence of blood, yields a blue reaction product within seconds.

Methodology

- The stool guaiac test involves fasting from iron supplements, red meat (the blood it contains can turn the test positive), certain vegetables (which contain a chemical with peroxidase properties that can turn the test positive), and vitamin C and citrus fruits (which can turn the test falsely negative) for some time before the test.
- It has been suggested that cucumber, cauliflower and horseradish, and often other vegetables should be avoided for three days before the test.
- In testing, feces are applied to a thick piece of paper attached to a thin film coated with guaiac.
- Either the patient or medical professional smears a small fecal sample onto the film.
- The fecal sample is obtained by catching the stool and transferring a sample with an applicator.
- Digital rectal examination specimens are also used but this method is discouraged for colorectal cancer screening due to very poor performance characteristics.
- Both sides of the test card can be peeled open, to access the inner guaiac paper. One side of the card is marked for application of the stool and the other is for the developer fluid.
- After applying the feces, one or two drops of hydrogen peroxide are then dripped on to the other side of the film, and it is observed for a rapid blue color change.

Disadvantages

- This test has low adherence rates due to dietary restrictions and the need for 3 different samples.
- Although specific, it has low sensitivity as it cannot detect Hb below 600 μg/g feces. Hence precursors of most CRCs can be missed.
- High sensitivity gFOBTs like HEMOCCULT SENSA II have been designed but these have lower specificity making this test less suitable for population-based screening.

Fecal Immunochemical Test

- It detects human globin using antibody-based assay.
- It is an FDA approved test for CRC screening done annually.
- FIT only detects human blood from the lower intestines.
- Medicines and food do not interfere with the test. So it tends to be more accurate and have fewer false-positive results than other tests.
- It is more sensitive than guaiac FOBT.

Molecular Carcinogenesis Pathways in CRC

- Chromosomal instability (CIN) pathway
- Microsatellite instability (MSI) pathway and
- CpG island methylator phenotype (CIMP) or epigenetic instability pathway.

Chromosomal Instability Pathway

- Chromosomal instability (CIN) is a type of genomic instability in which chromosomes are unstable, such that either whole chromosomes or parts of chromosomes are duplicated or deleted.
- They have altered total DNA content, cytogenetics, aneuploidy and numerous allelic gains and losses and translocations.
- It accounts for 84% of sporadic colorectal carcinoma cases.
- There is a combination of oncogene activation (e.g. KRAS, PIK3CA) and tumor suppressor gene inactivation (e.g. APC, SMAD4 and TP53) by allelic loss and mutation.
- APC is the most common gene that undergoes mutation and is implicated in familial adenomatosis polyposis and sporadic colorectal carcinoma cases.
- Both copies of APC must be lost either by genetic or epigenetic events. Loss of APC activates the beta-catenin (Wnt pathway) which causes uncontrolled proliferation.
- It occurs early in the adenoma-carcinoma sequence.

Microsatellite Instability Pathway

- Microsatellite instability (MSI) is the condition of genetic hypermutability (predisposition to mutation) that results from impaired DNA mismatch repair (MMR).
- It is involved in 10% to 15% of sporadic cases.
- There may be no detectable antecedent lesions, or the tumors may develop from sessile serrated adenomas.
- Inherited mutations in one of five DNA mismatch repair genes (MSH2, MSH6, MLH1, PMS1, and PMS2) give rise to the hereditary nonpolyposis colon carcinoma (HNPCC).
- Of these, MLH1 and MSH2 are the ones most commonly involved in HNPCC-derived and sporadic colon carcinomas with DNA mismatch repair gene defects.
- Loss of DNA mismatch repair genes leads to a hypermutable state in which simple repetitive DNA sequences, called ***microsatellites***, are unstable during DNA replication, giving rise to widespread alterations in these repeats.
- The resulting ***microsatellite instability*** (MSI) is the molecular signature of defective DNA mismatch repair, and hence this pathway is often referred to as the MSI pathway.
- Because of the multiple mutations caused as a consequence of the defect in DNA repair, the defect is considered to lead to the establishment of a mutator phenotype.
- Most microsatellite sequences are in noncoding regions of the genes.
- However, some microsatellite sequences are located in the coding or promoter region of genes involved in the regulation of cell growth.
- Such genes include type II TGF-β receptor and *BAX*. TGF-β signaling inhibits the growth of colonic epithelial cells and the *BAX* gene product causes apoptosis.
- Loss of mismatch repair leads to the accumulation of mutations in these and other growth-regulating genes, culminating in the emergence of colorectal carcinomas.
- Fully developed tumors that arise via the mismatch repair pathway do show some distinctive morphologic features, including proximal colonic location, mucinous histology, and infiltration by lymphocytes.

- In general, these tumors have a better prognosis than stage-matched tumors that arise by the *APC*/β-catenin pathway.

Epigenetic Instability Pathway

- In addition to genomic DNA instability being a common phenomenon in colorectal cancer, epigenetic instability also appears to be common in colorectal neoplasms.
- Some regions are enriched for CpG dinucleotides, called CpG islands, that are present in the 5′ region of approximately 50–60% of genes and are normally maintained in an unmethylated state.
- In cancers, many of these CpG islands become aberrantly methylated, and this aberrant methylation can be accompanied by transcriptional repression.
- The aberrant methylation of MLH1 occurs in >80% of sporadic MSI colorectal cancers.
- In addition, there is a subgroup of colorectal cancers that hypermethylate a high proportion of genes that may belong to a distinct subclass of colorectal cancers, termed CpG island methylator phenotype (CIMP).
- These tumors are defined by a high proportion of aberrantly methylated gene promoters that arise by distinct and unique mechanisms compared with non-CIMP tumors.
- The mechanism responsible for causing CIMP tumors is not known but this class of tumors commonly possesses mutant BRAF V600E.

RECENT COLORECTAL STOOL SCREENING TESTS

- DNA and RNA tests
  - Single genetic and epigenetic markers
  - Multi-marker panel
- Protein markers
- Microbiome-based markers.

History

- In malignancy, number of colonocytes exfoliated is 4–5 times greater than from normal tissue.
- Genetic and epigenetic changes prevent the destruction of these colonocytes due to increased cellular proliferation or reduced cell-cell or cell-basement membrane adhesions.
- Over the past 30 years, a wide array of single genetic and epigenetic changes that are detectable within fecal samples have been identified.
- Concurrently, multi-marker panels have been proposed.

Separation of Abnormal Genetic Material

- Human genetic material within fecal specimens is sparse, with a median concentration of 309 ng/g stool.
- This accounts for only 0.01% of the total genetic material in stool, the remaining portion includes genetic material from colorectal microbial flora, eukaryotic parasites, and undigested dietary remains.
- To maximize the sensitivity of detecting mutant DNA for a screen of CRC in an asymptomatic population, it is important to maximize the recovery of target DNA from stool.

- There are 2 methods of separation of genetic material—magnetic bead-based sequence-specific purification and acrylamide-gel method for DNA purification.

Magnetic Bead-Based Sequence-Specific Purification

- Sequence-specific DNA fragments are purified from total nucleic acid preparations by performing oligonucleotide-based hybrid captures.
- In the capture reaction: 300 µl of sample is added to equal volume of 6 mol/L guanidine isothiocyanate solution (GITC) which contains biotinylated sequence-specific oligonucleotides.
- Then streptavidin-coated magnetic beads are used to elute the DNA from this mixture.

Acrylamide-Gel Method for DNA Purification

- In this method target human DNA fragments are purified from total nucleic acid preparations by electrophoretically driving DNA through an affinity capture layer.
- This layer consists of human, sequence-specific capture probes immobilized within an acrylamide matrix.

DNA and RNA Tests

Single Genetic Markers

- Most commonly reported and well-studied single genetic markers are KRAS, p53 and APC. DCC is another genetic marker but it is not very well studied.

KRAS

- It is located on the short arm of chromosome 12.
- It encodes the K-ras guanosine triphosphate (GTP)-binding protein with a role in signal transduction for regulation of proliferation and differentiation.
- The KRAS gene is most commonly activated by point mutations.
- Molecular analyzes of KRAS mutations have revealed three hot spots, which encode residues either within the GTP-binding pocket or the enzymatic region essential for GTP hydrolysis.
- Mutations at these locations interfere with GTP hydrolysis which is essential to convert RAS into an inactive form.
- KRAS is thus trapped in its activated GTP-bound form, and the cell is forced into a continuously proliferating state.
- KRAS was the first molecular marker to be studied.
- Mutations to this gene are more common in lesions of the distal colon.
- In fecal specimens, this mutation has been detected in 35–42% of CRCs and approximately 50% of adenomas larger than 1 cm.

Limitations

- Non-specific and is detected in morphologically normal colonic mucosa, self-limiting hyperplastic polyps.
- It may be positive in benign, unrelated pathology such as pancreatic hyperplasia.

TP53

- Mutations in TP53 occur in the later stages of adenoma–carcinoma sequence.
- It is located on the short arm of chromosome 17.
- Mutations may occur at exons 5, 6, 7, or 8.
- Following mutation, the p53 protein is deregulated, resulting in an increased malignant progression.
- On fecal screening, such mutations have been identified in 50–70% of CRCs, up to 64% of severely dysplastic polyps, and 4–26% of adenomas.

APC

- The adenomatous polyposis coli (APC) gene is found on chromosome 5q21
- It encodes the APC "gatekeeper" protein that is responsible for the regulation of β-catenin, an inductor of growth-promoting genes in the Wnt signaling pathway.
- Also it has a role in the regulation of cell adhesion, interaction with microtubules for cell migration, and blockage of the cell cycle.
- Mutations to the APC gene occur early in the adenoma–carcinoma sequence.
- Mutation can occur anywhere along the first 1600 codons of the gene.
- In fecal screening mutations are reported to be detected in 20–82% of adenomas and 52–60% of CRCs.

DCC

- Deleted in colon cancer (DCC) gene is found on the long arm of chromosome 18 and under normal circumstances controls apoptosis.
- Though well studied in tissue specimens, its expression in fecal samples remains poorly researched and understood.
- In one study, on fecal analysis, mutations were reported in 70% of CRCs and up to 60% in adenomas.
- Further research in this area is required to elucidate the potential role of DCC as a molecular marker.

Other Additional Markers

Fecal Microsatellite Instability

- The most common fecal marker of MSI is Big Adenine Tract 26 (BAT-26).
- BAT-26—a locus of 26 repeated adenine nucleotides located in the MSH-2 mismatch repair gene.
- Alone this fecal marker has a sensitivity of 40% for proximal CRCs (right-sided cancers proximal to splenic flexure).
- It is more commonly used as part of a multitarget panel.

Methylation Markers

- In hypermethylation, identical residues in the regulatory regions of the particular gene are targeted by cancers, thus facilitating as a good screening test.
- Methylation patterns have been detected during the early stages of tumorigenesis.

- Methylation-based testing has been suggested to be sensitive in the detection of CRC.
- Increasing number of specific markers representing epigenetic signatures are being identified.
- Such fecal markers are attractive screening tools due to their high prevalence in early-stage neoplasia and predictability as assay targets on gene promoter regions.
- These fecal screening tests can additionally be used as early disease markers, prognostic indicators, and predictors of therapy response.

Single Epigenetic Markers

Vimentin

- Vimentin gene is responsible for coding an intermediate filament protein constituent that normally is not expressed in colonic epithelium.
- Methylated vimentin can be detected when captured using a methyl-binding domain (MBD) protein.
- Fecal analysis of methylated vimentin has yielded a sensitivity of 46% and a specificity of 90% in CRCs.
- It is currently available commercially in the United States as ColoSure(TM) but is not FDA approved.

NDRG4

- NDRG4 gene (tumor suppressor gene) on chromosome 16q21– q22.3 encodes for a protein of the same name, N-Myc downstream-regulated gene 4 (NMDRG-4).
- Hypermethylation of this gene is seen in both proximal and distal colon cancers.
- Methylation of this marker was assessed which yielded a sensitivity of 61% and specificity of 93% in CRCs.

Multimarker Panel

- To date, no single molecular marker has been identified that is expressed in all CRCs.
- The genetic heterogeneity of these lesions suggests that a panel of molecular markers may be better suited for screening purposes.
- Studies that combine genetic and epigenetic markers have shown a higher sensitivity for colorectal lesions, though often at the expense of specificity.

PreGen Plus

- This is the best-studied multi-marker panel which is now commercially available under the name PreGen Plus.
- It has been available in the United States since 2003.
- It includes 21-point mutations on K-ras, APC and p53 combined with BAT-26 and a DNA integrity assay (DIA) to detect abnormalities in the apoptosis pathway and detect L-DNA.
- It is not FDA approved.

Cologuard™

- It is an automated multitargeted stool DNA testing product that is intended for the qualitative detection of colorectal neoplasia-associated DNA markers and for the presence of occult hemoglobin in human stool.

- DNA biomarker detection is done by amplification and detection of methylated target DNA **(**NDRG4, BMP3**),** KRAS **point mutations, and** ACTB (a reference gene for quantitative estimation of the total amount of human DNA in each sample).
- Fecal immunochemical test determines the concentration of Hb in the sample.
- DNA analysis of stool samples using Cologuard™ may be considered medically necessary as a screening technique for colorectal cancer in average risk, asymptomatic individuals between the ages of 50 and 75 years when no other colorectal cancer screening has been performed during the recommended screening interval.

Advantages of Molecular Markers

- **Complete screen:** Fecal specimen contains a genetic material representative of the entire colon, whereas sigmoidoscopy reaches only the distal third.
- Small amount of specimen is required—minute quantities of DNA can be amplified using PCR.
- Greater patient compliance—neither dietary nor medicine restriction is required.
- Non-invasive and safe.

Disadvantages

- The greatest challenge in fecal screening for CRC remains identification and selection of a single or panel of molecular markers with a high sensitivity and specificity.
- Many targets and combinations of molecular markers have been identified; however, which ones to use and how many of them remain undefined.
- This is further complicated by the understanding that the more markers used, the higher the cost.

PROTEIN MARKERS

M2 Pyruvate Kinase

- The test is based on the detection of proteins in stool derived from neoplastic colonocytes
- A recently reported study using M2PK revealed a CRC sensitivity and specificity of 79% and 80%, respectively.

Calprotectin

- It is a non-cancer-specific protein marker.
- It is a calcium-binding protein in granulocytes, macrophages, and epithelial cells.
- Elevation of calprotectin occurs during intestinal inflammation, including inflammation caused by inflammatory bowel disease.
- Elevated fecal calprotectin in CRC is suggested to be due to neutrophil shedding from an ulcerated tumor into the intestinal lumen.
- A large Norwegian CRC screening trial evaluated calprotectin and reported a lower sensitivity and specificity than FIT.

HUMAN FECAL MICROBIOME-BASED MARKERS

- Recent studies suggest an important role for the gut microbiome in the development of CRC.

- Patients with CRC have a different gut microbiome than healthy subjects.
- *Streptococcus bovis* was the first bacteria used.

POINTS TO REMEMBER

- The guaiac FOBT was one of the first fecal tests used in colorectal cancer screening.
- However, fecal immunochemical test has higher sensitivity than guaiac FOBT.
- Cologuard™ a multi-target stool DNA test was approved by the FDA in the USA for CRC screening in 2014. They are more expensive than the FOBTs, and come with a relatively low specificity.
- Therefore, a sensitive single biomarker or panel of biomarker (stool) tests at affordable cost is much awaited.
- So far, no single protein stool marker has shown to be of adequate accuracy to be considered for population-based CRC screening. The use of protein makers in CRC screening, or combining protein markers with FIT, requires further research.
- Also, further identification of the gut microbiome could open up new possibilities in CRC screening strategies.

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15 Pathogenesis of Ovarian Carcinoma: Recent Advances

Naveen Kumar

INTRODUCTION

- Ovarian cancer is one of the most common cancers in India after carcinoma breast and carcinoma cervix.
- Worldwide it is the fifth most common malignancy and fourth in cancer mortality.
- The incidence rates are 5.4 and 8.0 per 100,000.

Distribution of Ovarian Cancers in India

- Epithelial: 75%
- Germ cell: 14.3%
- Stromal: 10.7%

Etiology and Risk Factors

Age

- Ovarian cancer rates increase with age.
- The median age at diagnosis is 63 years and the median age at death is 71 years.
- Age is an independent prognostic factor.

Reproductive Factors

Protective Factors

- Increasing parity and oral contraceptive use.
- Pregnancy and breastfeeding.
- Surgically-induced protective factors like tubal ligation, and bilateral salpingo-oophorectomy.

Risk Factors

- Early menarche and late menopause.
- High socioeconomic status is associated with an increased ovarian cancer risk and lower fertility.

Ovulation and Hormonal Factors

- The most commonly cited hormonal mechanism is the "**Incessant ovulation hypothesis".**
- It states that ovulation repeatedly traumatizes the ovarian surface epithelium, stimulates proliferation and predisposes the actively proliferating epithelium to malignant transformation.
- It was supported by epidemiologic observations of a direct correlation between the number of ovulations, i.e., the length of the reproductive years uninterrupted by pregnancies or oral contraceptive use and the risk of ovarian cancer.
- Most of these studies report a 50% risk reduction on oral contraceptives.

Inflammation

- Ovulation-induced surface damage.
- Retrograde menstruation-induced salpingitis.
- Introduction of foreign material through the vagina and uterine cavity.
- Evidence of a proinflammatory microenvironment in endometriosis supports this hypothesis for Type I tumors.
- High-grade serous carcinomas are associated with chronic salpingitis in 53% of cases.

Old Paradigm of Pathogenesis of Ovarian Cancer

The time honored concepts that have forged our views of ovarian carcinogenesis are as follows:

- The majority of ovarian carcinomas are high-grade serous carcinomas and therefore ovarian cancer is regarded as a single disease.
- It originates from the ovarian surface epithelium (mesothelium) which invaginates into the underlying stroma resulting in inclusion cysts that eventually undergo malignant transformation.
- Ovarian cancer spreads from the ovary to the pelvis, abdomen and distant sites.
- Based on these views, efforts at improving survival have focused on early detection of ovarian cancer, when it is still confined to the ovary.

Table 15.1: Differences between the two types of ovarian tumors

Type I tumors	*Type II tumors*
Low grade	High grade and grows rapidly and are highly aggressive neoplasms.
Arise from well-characterized precursor lesions [atypical proliferative (borderline) tumors and endometriosis].	Arise from intraepithelial carcinomas, the majority of which have been detected in the tubal fimbriae.
Usually presents as large mass confined to ovary (stage I neoplasms)	Presents in advanced stage (stages II–IV) in over 75% of cases.
Serous (invasive micropapillary serous carcinoma), endometrioid, mucinous, clear cell carcinomas.	Serous carcinoma (usual type of serous carcinoma). Endometrioid carcinoma, malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas.

- Unfortunately, these efforts have not been successful as evidenced by the fact that the overall survival for women with ovarian cancer has not changed over the last 50 years.
- The reasons for this are that the concepts of histogenesis on which these approaches are based, are flawed.
- Recent morphologic and molecular genetic studies have resulted in a paradigm shift.

New Model of Ovarian Cancer Pathogenesis

- Epithelial ovarian carcinomas are a heterogeneous group of cases.
- It is divided into two groups (type I and type II)
- Both these types have distinctive precursors and molecular features as shown in Table 15.2.

Table 15.2: Type I versus Type II ovarian carcinoma: Precursors and molecular features

	Common precursors	*Most frequent mutations*	*Chromosomal instability*
Type I tumors			
Low-grade serous carcinoma (LGSC)	APST, noninvasive LGSC	KIUS, BRAF	Low
Low-grade endometrioid CA	Endometriosis/ endometriotic cyst	CTNNBI, PTEN	Low
Clear cell carcinoma	Endometriosis/ endometriotic cyst	PIK3CÁ	Low
Mucinous carcinoma	APMT	KRAS	Low
Type II tumors			
High-grade serous carcinoma	p53 signature, STIC	TP53	High
High-grade endometrioid carcinoma	U	TP53	High
Undifferentiated carcinoma	U	U	Probably high
Carcinosarcoma	STIC	TP53	U

Origin of High Grade Serous Tumors

- The conventional view of the origin of serous tumor has been that they were derived from the ovarian surface epithelium or cortical inclusion cysts.
- It is based on the assumption that the precursors would be in ovaries.
- Fallopian tubes were not carefully examined but it is not only ovary that is involved in the pathogenesis.
- The origin could also be from the following:
  - STIC (serous tubal intra-epithelial carcinoma)
  - STIL (serous tubal intra-epithelial lesion)
  - SCOUT (secretory outgrowth).

Serous Tubal Intraepithelial Carcinoma (STIC)

- It is the earliest recognizable form of tubal high-grade serous carcinoma and the immediate precursor of invasive carcinoma of the fallopian tube.
- It is present in up to 60% of sporadic HGSC.
- It shares genetic abnormalities with invasive high-grade serous carcinoma.
- STICs and early invasive tubular carcinomas occurred in both familial and sporadic ovarian carcinomas.
- Presence of identical *TP53* mutations in STICs and concomitant ovarian HGSCs, indicates a clonal relationship between them.

Protocol for Suspected Ovarian Carcinoma

- All salpingo-oophorectomy specimens with a clinical history of prophylactic or risk-reducing salpingo-oophorectomy or a family history of ovarian carcinoma or BRCA mutation carrier should be carefully examined.
- Surface epithelium: The external surface of the ovary and fallopian tube should be handled as gently as possible.
- Rubbing or scraping it or allowing it to dry should be avoided.

Sampling for Microscopic Examination Ovaries

- Ovaries should be serially sectioned perpendicular to the long axis and submitted in toto.
- If the lesion is present, submit 1 representative section/cm, or in toto if it is a small lesion.

Fallopian Tubes

- **SEE-FIM: S**ectioning and **E**xtensively **E**xamining the **FIM**briated End protocol.
- Submit in toto, cross-sectioned except fimbriae, which are longitudinally sectioned.
- Section the fimbriated end longitudinally.
- Serial section the remaining tube at 2–3 mm intervals (Fig. 15.1).
- The examination thus conducted uncovered a high incidence (38%) of serous tubal intraepithelial carcinomas in the fallopian tube, but none in the ovaries.
- Furthermore, 80% of these carcinomas appeared exclusively in the fimbriated end of the fallopian tube, indicating that the fimbria is the preferred site of serous carcinogenesis.

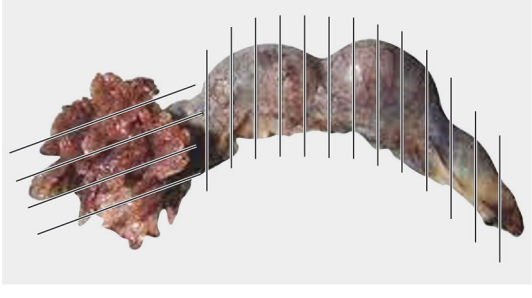

Fig. 15.1: SEE-FIM grossing protocol

WHY IS IT IMPORTANT?

Accurate diagnosis is very important because of the following:

- Extra-tubal spread may occur in the absence of invasion of the tube stroma.
- Clinicians may perform staging.
- Clinicians may recommend adjuvant chemotherapy.
- Serous tubal intraepithelial carcinoma (STIC) has a wide morphological spectrum.

Diagnostic Algorithm (Vang et al 2012)

Vang's diagnostic algorithm consists of three parameters:

1. Morphology
2. p53
3. Ki67

1. Morphology

Variable combination of histologic features:

- Nuclear enlargement,
- Hyperchromasia,
- Irregularly distributed chromatin,
- Nucleolar prominence,
- Mitotic activity,
- Apoptosis,
- Loss of polarity,
- Epithelial tufting.

Diagnostic Criteria of STIC

1. **Abnormal morphology** (either unequivocal or suspicious for STIC)
   1. *p53–abnormal pattern by IHC:* Diffuse nuclear moderate to strong expression in >75% of cells within the lesion or complete absence of expression within the lesion.
   2. *Ki67–high nuclear expression by IHC:* >10% positive cells within lesion.

Significance of p53 Mutation

- The detection of TP53 mutations in tubal intraepithelial carcinomas and described as putative precursor lesion is designated '**p53 signature.**'
- This lesion appears morphologically normal but overexpresses p53 and can harbor a p53 mutation.
- These findings highlight the importance of p53 mutation in the early development of high-grade serous carcinoma.

p53 Signature

- **A p53 signature is defined as a discrete expansion of at least 12 secretory-type cells with intense p53 nuclear staining.**
- Benign appearing non-ciliated tubal epithelium with overexpression of p53 but no increased proliferation (Fig. 15.2).

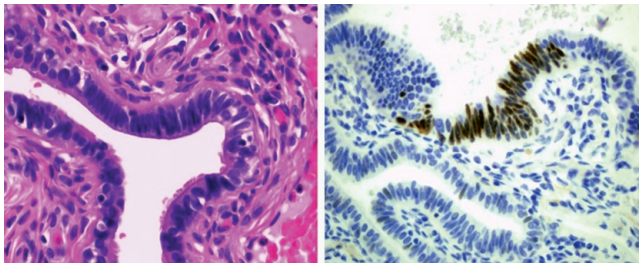

Fig. 15.2: p53 signature showing intense nuclear staining in tubal epithelium

- Most frequent in the distal tube and may represent a latent cancer precursor, but is of no known clinical significance, and is usually not reported when found.

Serous Tubal Intraepithelial Lesion (STIL)

- STIL is presumed to be a cancer precursor with abnormalities not sufficient for a diagnosis of STIC.
- It is less common and the clinical significance is uncertain.
- Diagnosis requires application of the diagnostic algorithm which usually does not fulfil all three criteria for STIC.
- A diagnosis of STIL should be accompanied by a description of the abnormal features and a comment indicating that the features are insufficient for a diagnosis of STIC.

Secretory Outgrowth (SCOUT)

- A SCOUT is defined as a discrete expansion of at least 30 epithelial cells of secretory type (BCL2 expressing, p73 non-expressing) that were distinct against a heterogeneous background of tubal secretory and ciliated cells.
- This is not limited to the distal tube and are seen more frequently in association with High-grade serous carcinoma.
- SCOUT is of no known clinical significance yet and is usually not reported.

BRCA Mutation in High-Grade Serous Carcinoma

- Inactivation of BRCA 1 and BRCA 2 by either mutation or hypermethylation is seen in type II ovarian HGSC.

Are STICs the only precursors?

- STICs were identified in ~50–60% of cases in various studies.
- **Possibilities:**
  - Small STICs were missed despite complete sampling of the tubes.
  - Carcinoma developed from ovarian cortical inclusion cysts.

Cortical Inclusion Cysts: Ovarian Epithelium or Fallopian Tube Epithelium?

Origin of Low-Grade Serous Carcinoma

- It arises from cortical inclusion cyst from the tubal epithelium and forms cystadenoma and then borderline tumors which eventually progress to low-grade serous carcinoma.
- These low-grade serous carcinomas will have KRAS and BRAF mutations.
- It also arises from fallopian tubes in women with atypical proliferative serous tumors (APST) which was classified as serous borderline tumor in previous classification.
- Papillary clusters of bland-appearing tubal epithelium that bud off, pass through the tube and implant on the ovary to develop into APST.

Non-invasive Micropapillary Serous Carcinoma

- It is an old term that has been changed to SBT-micropapillary variant/non-invasive low-grade serous carcinoma.

ATYPICAL PROLIFERATIVE SEROUS TUMORS (APST)/SBT

- Tumors at the lower end of the spectrum behave in a benign fashion and display a papillary architecture in which papillae have a hierarchical branching pattern.
- These tumors are termed 'APST'.
- APSTs comprise about 50% of atypical proliferative (borderline) ovarian tumors of all histologic types.

SBT-Micropapillary Variant/Noninvasive LGSC

- Tumors at the upper end of the spectrum behave like low-grade carcinomas.
- They display a more complex non-hierarchical branching pattern.
- They are characterized by delicate micropapillae, and are classified as non-invasive MPSC.

Clear Cell and Endometrioid Carcinoma

- Clear cell and endometrioid ovarian carcinomas have similar precursors and molecular genetic features.

Endometriosis and Ovarian Tumors

- Endometriosis is a common gynecologic disorder usually defined as the presence and/or growth of endometrial tissue outside the uterine cavity.
- The possibility of transformation of endometriotic tissue into malignant ovarian cancer has been recognized for almost 100 years.
- In 1925, Sampson first described malignant changes in endometriosis and proposed the criteria:
  - Coexistence of carcinoma and endometriosis within the same ovary,
  - A similar histological pattern,
  - Exclusion of a second malignant tumor elsewhere.
- In 1953, Scott postulated that in addition to the criteria by Sampson, morphological changes demonstrated by benign endometriosis that are contiguous with malignant tissue is a prerequisite for malignancy originating from endometriosis.
- Histopathological studies have assumed atypical endometriosis to be a transition between benign endometriosis and cancer.
- This suggests the possibility that endometriosis is in fact a premalignant condition.
- It is supported by the fact that 60–80% of endometriosis-associated ovarian carcinoma occurs in the presence of atypical ovarian endometriosis.
- Sato et al. demonstrated somatic mutations of PTEN in 21% of ovarian endometriotic cysts, indicative of shared molecular alterations between clear cell and endometrioid carcinomas of the ovary and their putative precursor lesion.
- Ovarian endometriosis (including endometriomas/endometriotic cysts) is far more common than benign endometrioid neoplasms.
- The revised WHO classification includes endometriotic cysts in the category of benign endometrioid tumors.

Molecular Evidence

- Mutations in **ARID1A** and **PIK3CA** were first described in numerous cases of ovarian clear cell carcinoma, but later also in precursor endometriotic lesions.

- Mutation of *ARID1A* in ovarian clear cell carcinoma and endometrioid carcinoma was first reported in 2010.
- ARID1A is located on chromosome 1p36.11 and encodes ARID1A, a core member of the SWI/SNF complex.
- ARID1A mutations occur in ovarian cancers at high frequency, and a close association with endometriosis-associated ovarian cancer has been shown.
- ARID1A mutations produce abnormalities in chromatin remodeling and contribute to carcinogenesis from endometriosis to endometriosis-associated ovarian cancers.
- ARID1A mutation is found in 46–57% of ovarian clear cell carcinomas.
- Other mutations in KRAS, BRAF, and TP53 are present in some clear cell carcinomas but their frequency is generally low.

Endometrioid Carcinoma

- The vast majority of endometrioid ovarian neoplasms are carcinomas.
- Endometrioid adenofibromas and atypical proliferative endometrioid tumors are uncommon.
- Molecular biological studies have shown that endometrioid ovarian carcinomas have similarities with and differences from their uterine counterparts.
- ARID1A mutation is found in 30% of endometrioid carcinomas.
- Apart from this Wnt (i.e., Wnt/β-catenin) signaling pathway is involved in the regulation of several important cellular processes, including cell fate determination, proliferation, motility and survival.
- Wnt/β-catenin-mediated signaling is deregulated in 16–38% of ovarian endometrioid carcinomas, usually based on activating mutations of CTNNB1.
- Also inactivating mutations in genes encoding negative regulators of β-catenin such as APC, AXIN1, or AXIN2 are reported rarely.
- Inactivating mutations of PTEN have been reported in 14–21% of ovarian endometrioid carcinomas.
- PIK3CA mutations were identified in 20% of ovarian endometrioid and clear cell carcinomas but in only 2% of ovarian serous carcinomas.
- Akin to the serous carcinomas, the molecular findings support the division of ovarian endometrioid carcinomas into two subgroups.
- Low-grade tumors are characterized by mutations that deregulate the canonical Wnt/β-cat and PI3K/PTEN signaling pathways and typically lack TP53 mutations which is a type I.
- High-grade tumors often harbor mutations of TP53 and lack Wnt/β-cat or PI3K/PTEN signaling pathway defects are seen in type II.

Recent Interest in MSI

- Microsatellites are segments of DNA in which a short sequence motif, usually 1 to 5 nucleotides in length, repeated several times.
- MSI refers to alterations in the DNA of tumor cells in which the number of sequence repeats within these microsatellites is different from the patient's non-neoplastic cells.
- A recent meta-analysis estimates the frequency of MSI phenotype in ovarian cancers at approximately 12%.
- 13–20% of ovarian endometrioid carcinomas are usually associated with mismatch repair, particularly hMLH1 and less frequently, hMSH2.

MUCINOUS TUMORS

- Mucinous tumors comprise about 7 to 15% of epithelial ovarian tumors of which 77% are metastasis to the ovary and 23% are primary ovarian mucinous tumors.
- In primary ovarian mucinous carcinomas, KRAS mutation is a very common molecular alteration occurring in ~75% of ovarian mucinous carcinomas.
- Identical KRAS mutations in mucinous carcinoma and adjacent mucinous cystadenoma and mucinous borderline tumors/atypical proliferative mucinous tumors have been observed.
- This molecular finding supports this morphological continuum of tumor progression in ovarian mucinous neoplasia.
- Hence, their classification as Type I tumors in the dualistic model of ovarian cancer pathogenesis.

Origin of Mucinous and Brenner Tumors

- Mucinous metaplasia involving cortical inclusion cysts is a rare finding.
- Mucinous cystadenomas contain foci of Brenner tumor in 18% of cases.
- Mucinous tumors are frequently associated with Walthard cell nests that are composed of benign transitional-type epithelium.
- Hence, the possibility that mucinous tumors and Brenner tumors have the same histogenesis.
- As a small Brenner tumor grows, the mucinous component becomes dominant resulting in the development of a mucinous cystadenoma.
- As it enlarges, compresses and eventually obliterates the adjacent ovary and Brenner tumor, gives the appearance that it arose in the ovary.

Targeted Therapy based on New Molecular Pathogenesis

- **Type I tumors** harbour KRAS and BRAF mutations-targeted therapy as MEK inhibitors—selumetinib, binimetinib under clinical trials.
- **Type II tumors:** High level of genetic instability and lack of shared aberrations of cellular signaling pathways.
- Recently ***Olaparib, a PARP [poly (ADP-ribose) polymerase] inhibitor***, received USFDA approval in 2014 for fourth-line treatment of recurrent ovarian cancer in women with germline BRCA mutations.
- With this targeted therapy, PARP is trapped at sites of DNA damage and results in the formation of a PARP-DNA complex causing cell death in tumors that are deficient in HRR pathways.
- More recently, ***olaparib*** was approved for the maintenance treatment of recurrent platinum-sensitive disease.
- Other useful PARP inhibitors include ***rucaparib***, which was approved in 2016 for third-line treatment of BRCA-mutated ovarian cancers.
- **Niraparib**, was approved in 2017 for maintenance treatment of recurrent platinum-sensitive disease.
- The vascular endothelial growth factor (VEGF) inhibitor, bevacizumab, has activity in ovarian cancer and is USFDA-approved for recurrent disease.
- Finally, immune checkpoint inhibitors such as agents targeting the PD-1/PD-L1 pathway of immune regulation may provide new opportunities for the treatment of ovarian cancer.

- A recent study found that PD-L1 expression in ovarian carcinomas is predominantly confined to tumor-associated macrophages.
- Several clinical trials of these agents in ovarian cancer are currently in progress.

POINTS TO REMEMBER

- There is a new paradigm shift in ovarian carcinoma pathogenesis with molecular evidences.
- Careful examination of fallopian tubes for STIC, STIL and SCOUT lesions in high-risk patients is important.
- ARID1A is the new genetic mutation in ovarian clear cell and endometrioid carcinoma.
- These new molecular pathogenetic pathways provided newer modalities for targeted therapy for ovarian carcinomas.

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16 Recent Advances in Understanding of Renal Tumors

Tanisha Singla

INTRODUCTION

- Kidney cancer is the ninth most common cancer and the 16th leading cause of cancer death worldwide.
- It is much less common in Southeast Asian countries with 1% incidence.
- It is more common in men than women (approximately 2:1) and is rare in children.

Risk Factors

- Obesity
- Smoking
- Hypertension
- Acquired cystic kidney disease in a long-term hemodialysis patient due to end-stage renal disease is an important risk factor. Acquired cystic kidney disease-associated RCC (new entity) has replaced renal papillary carcinoma as the most common cancer associated with it.
- Trichloroethylene which is a solvent used as a metal degreaser and chemical additive is a significant risk factor.
- Although most renal carcinomas are sporadic, 2–4% have a familial cause.
- Several genetic diseases are associated with renal cancer shown in Table 16.1.

Table 16.1: Hereditary renal cell tumours

Syndrome	*Protein*	*Tumor type associated*
von Hippel-Lindau syndrome	VHL protein	Multiple bilateral clear cell RCC
Hereditary leiomyomatosis and RCC	Fumarate hydratase	Papillary renal cell carcinoma (non-type 1)
Hyperparathyroidism-Jaw tumor syndrome	Parafibromin	Mixed epithelial and stromal tumor, papillary RCC
Birt-Hogg-Dube syndrome	Folliculin	Multiple chromophobe RCC, papillary RCC
Tuberous sclerosis	Hamartin, tuberin	Bilateral angiomyolipoma

WHO 2016 CLASSIFICATION

- A few new subtypes have been added in the last 2016 WHO classification (Table 16.2).
- They have been named based on predominant cytoplasmic features and staining characteristics (e.g. clear cell and chromophobe RCCs), architectural features (e.g. papillary RCC), cell type (e.g. renal oncocytoma), and combinations of these features (e.g. clear cell papillary renal cell carcinoma).
- Other approaches that were used include resemblance to embryological structures such as the metanephros (e.g. metanephric adenoma), anatomical location of the tumors (e.g. collecting duct and renal medullary carcinomas), and a correlation with background renal disease (e.g. acquired cystic disease-associated renal cell carcinoma).
- Names referring to molecular alterations (e.g. MiT family translocation carcinomas and succinate dehydrogenase-deficient renal cell carcinoma) have been added.
- Familial predisposition has also been denoted (e.g. hereditary leiomyomatosis and renal cell carcinoma -associated renal cell carcinoma).

Table 16.2: Updated 2016 WHO classification for renal tumours

Renal cell tumours	
Clear cell renal cell carcinoma	Succinate dehydrogenase-deficient renal cell carcinoma**
Multilocular cystic neoplasm of low malignant potential*	Mucinous tubular and spindle cell carcinoma
Papillary renal cell carcinoma	Tubulocystic renal cell carcinoma**
Hereditary leiomyomatosis and renal cell carcinoma- associated renal cell carcinoma**	Acquired cystic disease-associated renal cell carcinoma**
Chromophobe renal cell carcinoma	Clear cell papillary renal cell carcinoma**
Collecting duct carcinoma	Renal cell carcinoma unclassified
Renal medullary carcinoma	Papillary adenoma
MIT family translocation renal cell carcinomas*	Oncocytoma
Metanephric tumours	
Metanephric adenoma	Metanephric stromal tumour
Metanephric adenofibroma	
Nephroblastic and cystic tumours occurring mainly in children	
Nephrogenic rests	Cystic partially differentiated nephroblastoma**
Nephroblastoma	Pediatric cystic nephroma***
Mesenchymal tumours	
Occurring mainly in children	
Clear cell sarcoma	Ossifying renal tumour of infancy
Rhabdoid tumour	Congenital mesoblastic nephroma

Contd.

Table 16.2: Updated 2016 WHO classification for renal tumours	
Occurring mainly in adults	
Leiomyosarcoma	Leiomyoma
Rhabdomyosarcoma	Hemangioma
Angiosarcoma	Lymphangioma
Osteosarcoma	Hemangioblastoma
Ewing's sarcoma	Juxtaglomerular cell tumour
Synovial sarcoma	Renomedullary interstitial cell tumour
Angiomyolipoma	Schwannoma
Epithelioid angiomyolipoma**	Solitary fibrous tumour
Mixed epithelial and stromal tumour family	
Adult cystic nephroma***	Mixed epithelial and stromal tumour
Neuroendocrine tumours	
Well-differentiated neuroendocrine tumour	Small cell neuroendocrine carcinoma
Large cell neuroendocrine carcinoma	Paraganglioma
Miscellaneous tumours	
Renal hematopoietic neoplasms	Germ cell tumours
Metastatic tumours	

*Indicates change of terminology
**Newly added entities
***Paediatric cystic nephroma has been separated from adult cystic nephroma

EMERGING/PROVISIONAL ENTITIES

- The 2013 International Society of Urological Pathology (ISUP) Vancouver Classification of Renal Neoplasia identified a category of emerging or provisional new entities (Table 16.3).
- Though these entities appeared to be distinct, these rare tumors were not yet fully characterized by morphology, immunohistochemistry, and molecular studies.
- Further reports were needed to refine their diagnostic criteria and establish clinical outcomes.

Table 16.3: Emerging/provisional renal cell carcinomas
Oncocytic renal cell carcinoma occurring after neuroblastoma
Thyroid-like follicular renal cell carcinoma
ALK rearrangement associated renal cell carcinoma
Renal cell carcinoma with angioleiomyomatous stroma

TUMOUR GRADING

- Earlier Fuhrman system was the most widely used. However, there were problems with the interpretation, validation, and reproducibility of the Fuhrman system
- For these reasons, the four-tiered WHO International Society of Urological Pathology (ISUP) grading system should be used (Table 16.4).

- It has not yet been validated as a prognostic marker for other morphological types of renal cell neoplasia (primarily due to a small number of reported cases).

Table 16.4: WHO/ISUP grading for clear cell carcinoma and papillary renal cell carcinoma

Grade	*Description*
Grade 1	Nucleoli are absent or inconspicuous and basophilic at x400 magnification
Grade 2	Nucleoli are conspicuous and eosinophilic at x400 magnification and visible but not prominent at x100 magnification
Grade 3	Nucleoli are conspicuous and eosinophilic at x100 magnification
Grade 4	There is extreme nuclear pleomorphism, multinucleate giant cells and/or rhabdoid, and/or sarcomatoid differentiation.

CLEAR CELL RENAL CELL CARCINOMA

- It is a morphologically heterogeneous group of malignant neoplasms composed of cells with clear or eosinophilic cytoplasm.
- These neoplasms have typical vessel formation and a characteristic molecular background, with inactivation of VHL and upregulation of hypoxia-inducible factor.
- Classical triad includes hematuria, flank pain and abdominal mass and is seen in 9% cases.
- VHL is a tumor suppressor gene located on 3p25 and is altered in both sporadic and hereditary clear cell renal cell carcinomas.
- There is a 2 hit hypothesis for tumorigenesis.
- Individuals with VHL disease are born with one inactivated copy of the VHL gene in all cells while the other copy of the gene is normal.
- 'Second hit' usually takes place as a result of somatic mutation or deletion of the allele.
- In patients with sporadic renal cell carcinoma, inactivation of both VHL alleles usually takes place via somatic mutations.
- PD1-PD-L1 also plays an important role in the pathogenesis of renal cell carcinoma.
- PD-L1 is expressed on cancer cells and it combines with PD-1 receptor on cytotoxic T lymphocytes to cause their apoptosis.

Morphology

- It is a well-demarcated tumor separated by a pseudocapsule.
- The cut surface is solid, golden yellow with areas of hemorrhage, cystic change giving it a characteristic variegated appearance.
- Microscopically, tumor cells are arranged in the form of a nesting pattern separated by stroma consisting of prominent sinusoid-like vessels (Fig. 16.1).

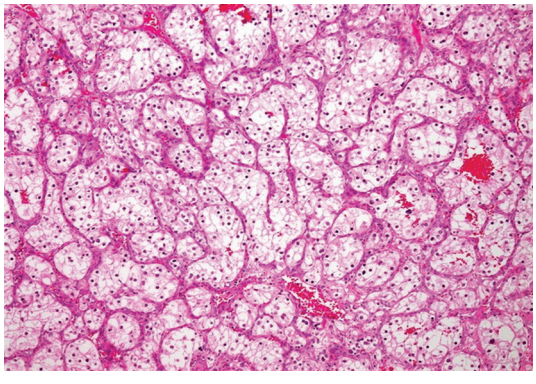

Fig. 16.1: Nests of clear cells in clear cell RCC

- Cells are large with cytoplasmic appearance ranging from optically clear cytoplasm with a well-defined cell boundary to granular cytoplasm.
- Immunohistochemically, co-expression of CK and vimentin is the rule.
- Other markers expressed are EMA, CEA, alpha1 antitrypsin, ACE, CD10, CA IX, PAX 2, PAX 8, VHL gene product.

DRUGS

- VEGF inhibitors are first-line therapy for the treatment of metastatic clear cell carcinoma.
- Sunitinib and pazopanib are two commonly used tyrosine kinase inhibitors.
- PD-1-blocking antibodies currently available are nivolumab and pembrolizumab.
- Nivolumab is currently approved for the treatment of metastatic clear cell carcinoma in the second-line setting.

Multilocular Cystic Renal Neoplasm of Low Malignant Potential

- It was earlier known as multilocular cystic renal cell carcinoma.
- It is a tumor composed entirely of numerous cysts, the septa of which contain individual or groups of clear cells without expansile growth.
- It has an excellent prognosis with no recurrence or metastases.
- The genetic profile is similar to clear cell carcinoma showing a loss of 3p.

Morphology

- A well-circumscribed multicystic mass is seen separated by a fibrous pseudocapsule.
- Variable-sized cysts are seen filled with serous or bloody fluid with the absence of solid masses (Fig. 16.2).
- Microscopically, the variable sized cysts are lined by a single layer of small bland cells without nucleoli with clear cytoplasm.
- The septa consist of fibrous tissue with calcification or ossification along with clusters of tumor cells (without expansile growth) similar to those lining the cysts.
- No areas of necrosis or sarcomatoid change is seen (Fig. 16.3).

Fig. 16.2: A well-circumscribed multicystic mass with multiple variably sized cysts filled with haemorrhagic fluid

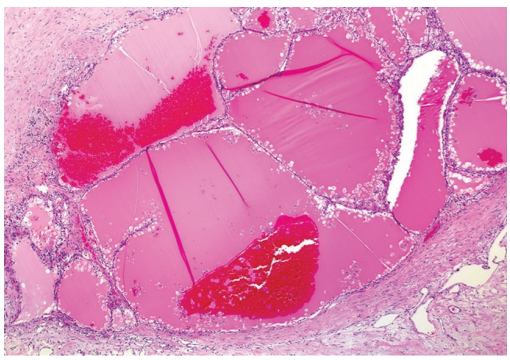

Fig. 16.3: Variable sized cysts lined by a single layer of small bland cells without nucleoli and clear cytoplasm. Tumour is separated by a fibrous pseudocapsule

Clear Cell Papillary Renal Cell Carcinoma

- The tumour is composed of clear cells of low nuclear grade.
- Variable papillary, tubular, or acinar patterns can be seen.
- It is a clinically indolent low-stage tumor.
- On a small core biopsy, this tumour can be mistaken for clear cell renal cell carcinoma.
- It lacks genetic abnormalities seen in clear cell and papillary renal cell carcinoma.
- There is an activation of the HIF pathway by non-VHL-dependent mechanisms.
- Nuclei show pseudoendometrial appearance.
- Typical IHC profile is CA IX, CK7, cytokeratin 34betaE12, Vimentin, HIF-1 and GLUT-1 positive with CD10 and AMACR negative.

HLRCC Associated Renal Cell Carcinoma

- This tumour results from germline fumarate hydratase (FH) mutations.
- Deficient fumarate hydratase leads to an increase in fumarate which is an oncometabolite.
- Leiomyomas of the skin and uterus are the other most common features.
- It has a poor prognosis.
- Microscopically, they show papillary, tubular, tubulopapillary and solid architectural patterns.
- The tumour cells are large with abundant cytoplasm.
- Nuclear features are characteristic showing large nuclei with an eosinophilic nucleolus surrounded by a clear halo.
- Overexpression for 2-succino-cysteine, is more sensitive and specific for FH gene mutation than loss of FH by IHC.
- The staining is both nuclear and cytoplasmic.

Collecting Duct Carcinoma

- It is also known as Bellini duct carcinoma.
- Being a highly aggressive tumor, 50% cases present with metastasis and has a poor prognosis.
- There are updated diagnostic criteria for collecting duct carcinoma as shown in Table 16.5.

Table 16.5: Diagnostic criteria for collecting duct carcinoma

1. Medullary involvement
2. Predominant tubular morphology
3. Desmoplastic stromal reaction
4. Cytologically high-grade
5. Infiltrative growth pattern
6. Absence of other renal cell carcinoma subtypes or urothelial carcinoma

MIT FAMILY TRANSLOCATION CARCINOMAS

- This class of tumours harbor gene fusions involving members of the MiT family of transcription factors, including TFE3 and TFEB.
- Prior exposure to cytotoxic chemotherapy is the only risk factor.

- Xp11 translocation renal cell carcinoma involves fusion between ASPL-TFE3 gene due to balanced translocation (X;17).
- It accounts for approximately 40% of paediatric renal cell carcinomas. The prognosis is similar to clear cell RCC but worse than papillary RCC.
- Microscopically, typically nested pattern is seen with epithelioid clear to eosinophilic cells, hyaline globules and abundant psammomatous calcifications with voluminous cytoplasm.
- IHC-nuclear positivity for TFE3.
- It can be confirmed by break-apart TFE3 FISH.
- t(6;11) Renal cell carcinoma involves fusion of TFEB-MALAT1(alpha).
- It is less common than the Xp11 translocation RCCs; only approximately 50 cases have been reported in the literature worldwide.
- Microscopically, it is a biphasic nested epithelioid cell tumor featuring larger epithelioid cells with clear to eosinophilic cytoplasm and clusters of smaller cells clustered around hyaline nodules.
- IHC-patchy positive for HMB45 and nuclear positivity for TFEB is seen. (d/d- Xp11 translocation RCC which is negative).
- It is morphologically distinct from Xp11 translocation RCC but can show overlap and is more indolent.
- It can be confirmed by break-apart TFEB FISH.

Succinate Dehydrogenase Deficient Renal Cell Carcinoma

- It is a rare tumor that occurs in young adults.
- These tumors show a germline mutation (double hit inactivation) in an SDH gene, usually SDH B (less commonly SDH C and rarely SDH A and D) leading to dysfunction of mitochondrial complex II.
- Personal or family history of paragangliomas, SDH deficient gastrointestinal stromal tumor, or pituitary adenoma may be present.
- Majority (75%) of cases are uniformly low-grade, lack coagulative necrosis, and have a favourable prognosis.
- Grossly, it is a well-circumscribed, solid tumour with red / brown cut surface, variable multicystic change can be seen.
- Microscopically, a well-circumscribed, lobulated tumour with pushing margin, often entrapping benign tubules is seen.
- Solid, nested, or tubular growth pattern is seen with scattered cysts containing eosinophilic material.
- Neoplastic cells have smooth nuclear contours and fine chromatin with no nucleoli. The characteristic finding is flocculent cytoplasmic vacuoles with a pale eosinophilic, wispy or bubbly appearance with low-grade nuclei.
- Loss of SDHB immunohistochemistry is seen in the tumor cells, whereas the entrapped tubules serve as the internal control.

Tubulocystic Carcinoma

- It is also known as low grade collecting duct carcinoma.
- Typically arises from renal cortex or corticomedullary junction.
- It is often seen as an incidental finding, usually low stage
- Prognosis is excellent.

- Grossly, it is a well-circumscribed tumor with a spongy cut surface and variable sized cysts giving a typical bubbly appearance.
- Microscopically, a mixture of closely packed tubules and micro/macro cysts of variable sizes are seen.
- Tubules and cysts are lined by a single layer of cuboidal or columnar cells with abundant eosinophilic cytoplasm, irregular nuclei with distinct grade 3 nucleoli; often having a hobnail appearance.
- There is minimal mitotic activity, no atypia, and fibrotic stroma.
- **Differential diagnosis**: Multilocular cystic renal neoplasm of low malignant potential—clear cells line the cysts with no formation of tubules.

Acquired Cystic Disease-Associated Renal Cell Carcinoma

- This tumour arises in kidneys with acquired cystic disease due to end-stage renal disease.
- Most tumours have indolent behavior and rarely can have sarcomatoid change.
- They typically do not express CK7 but are positive for CD10 and AMACR.
- It is associated with gains of the Y chromosome (distinguishes from papillary RCC).
- Microscopically shows a variety of growth patterns—acinar, alveolar, tubular, multicystic, papillary and solid microcystic architecture.
- Cells are polygonal to columnar with abundant eosinophilic to clear cytoplasm, round and central nuclei, finely granular chromatin, prominent central grade 3 nucleoli.
- Intratumoral calcium oxalate crystals can be seen.

Oncocytoma

- It is a benign renal epithelial neoplasm that is more common in males than females.
- It presents as an asymptomatic mass and closely resembles an eosinophilic variant of the chromophobe renal cell carcinoma.
- Frequent genetic abnormalities are loss of chromosome 1 and Y.
- Grossly, it is well-circumscribed, non-encapsulated tumor.
- Cut surface is mahogany brown with a central stellate radiating scar with no areas of hemorrhage and necrosis. (Fig. 16.4).
- Microscopically, tumor cells are arranged in solid nests mainly.
- They can be arranged in acini, tubules in a hypocellular stroma.
- Cells are round to polygonal with granular eosinophilic cytoplasm, round regular nuclei with evenly distributed chromatin and a central nucleolus (Fig. 16.5).
- Occasionally cluster of cells show pleomorphism with hyperchromatic nuclei and no atypical mitoses—degenerative changes.

Fig. 16.4: Well-circumscribed tumor, cut surface is mahogany brown with a central stellate scar

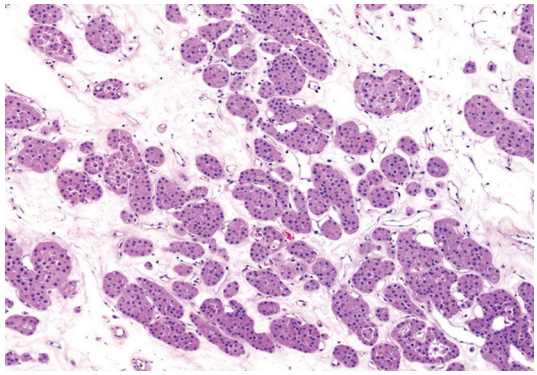

Fig. 16.5: Tumour cells arranged in solid nests. The cells have abundant granular eosinophilic cytoplasm and round regular nucleus

WILMS TUMOR (NEPHROBLASTOMA)

- It is the most common pediatric tumor with a peak incidence between 2 and 5 years.
- Clinically, it presents as an abdominal mass felt by the parents.
- It is associated with several dysmorphic syndromes like WAGR syndrome, Beckwith-Wiedemann syndrome, Denys-Drash syndrome.
- Grossly, it is well-circumscribed, solitary, rounded tumor and soft in consistency.
- Cut surface is solid homogenous, pale gray or tan (Fig. 16.6).
- Microscopically, it can be triphasic, biphasic, or monophasic. It is composed of blastemal, epithelial and stromal components.

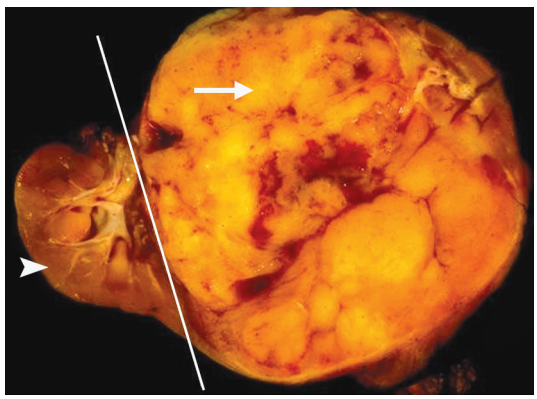

Fig. 16.6: Solid homogenous solitary tumour

- Blastemal component is cellular, composed of small round to oval primitive cells, scant cytoplasm, round to oval nuclei with inconspicuous nucleoli.
- Tumor cells can be arranged in a diffuse, nodular, serpentine (cord like) and basaloid with peripheral palisading.
- Epithelial component is composed of embryonic (abortive) glomerular and tubular structures resembling normal kidney.
- Stromal component usually has a spindle cell fibroblast-like configuration (Fig. 16.7).
- Tumors with anaplasia are said to have unfavorable histology since they show a lesser response to chemotherapy.
- Three histologic criteria have to be met to classify tumor as having unfavorable histology:
  1. Nuclei enlarged to at least 3 times the size of adjacent nuclei of the same cell type.
  2. Marked hyperchromasia of the enlarged nuclei.
  3. Multipolar mitotic figures (Fig. 16.7).

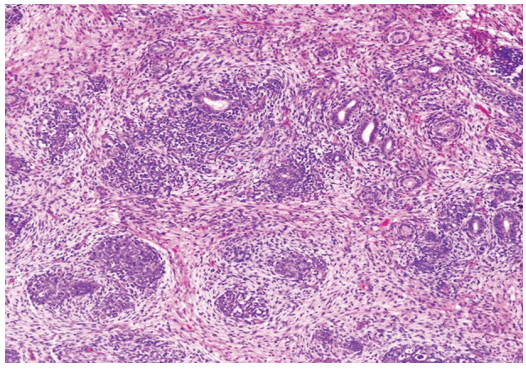

Fig. 16.7: Triphasic tumour composed of blastemal, epithelial and stromal component

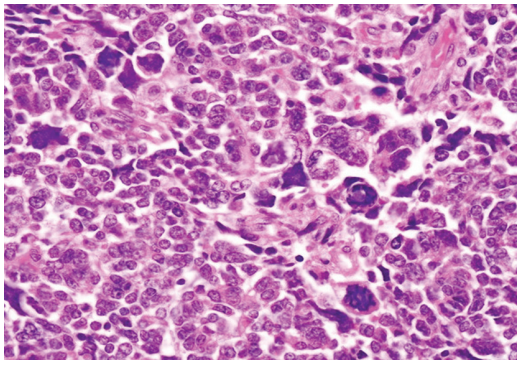

Fig. 16.8: Tumour with anaplastic foci

- Anaplasia is of two types—focal and diffuse (Table 16.6).

Table 16.6: Focal vs diffuse anaplasia

Focal anaplasia	*Diffuse anaplasia*
Anaplasia confined to one or more discrete sites within the primary tumor and not present in extrarenal sites	Anaplastic change or nuclear unrest in invasive or extrarenal deposits
Tumor cells outside anaplastic foci show no *nuclear unrest*.	Anaplasia beyond the tumor capsule
Anaplasia is not present within vascular spaces	Anaplasia not clearly delimited from non-anaplastic tumor Anaplasia in a random biopsy specimen
	Anaplasia not meeting the criteria for focal anaplasia

Cystic Partially Differentiated Nephroblastoma Vs Paediatric Cystic Nephroma

- Traditionally, both these tumors were considered to be part of cystic nephroblastoma.
- Both these tumors are well-encapsulated, composed entirely of cystic spaces and delicate septa without an expansile solid component.
- Cysts in both lesions are lined by flattened to columnar epithelium which is often hobnail type
- Both are curable by resection alone.
- Pediatric cystic nephroma consists of DICER1 mutation which is absent in cystic partially differentiated nephroblastoma.

Angiomyolipoma

- It is a distinct neoplasm consisting of a variable amount of smooth muscle, adipose tissue and vasculature.
- It can occur in extrarenal sites such as liver, lungs, retroperitoneal soft tissue.
- It may be sporadic but is associated with tuberous sclerosis.

- It is a member of perivascular epithelioid cell (PEC) tumor family, which includes pulmonary lymphangioleiomyomatosis, clear cell sugar tumors of pancreas, lung and uterus and cardiac rhabdomyoma.
- Grossly, it is well-circumscribed with pushing margins.
- Cut surface shows variable smooth muscles and adipose tissue and also vessels.
- Areas with adipose tissue are yellow and soft in consistency, whereas areas with smooth muscle are firm and white in appearance (Fig. 16.9).
- Microscopically, the tumor is triphasic with myoid spindle cells.
- Islands of mature adipose tissue and hyalinized thick-walled blood vessels with eccentric lumen can be seen.

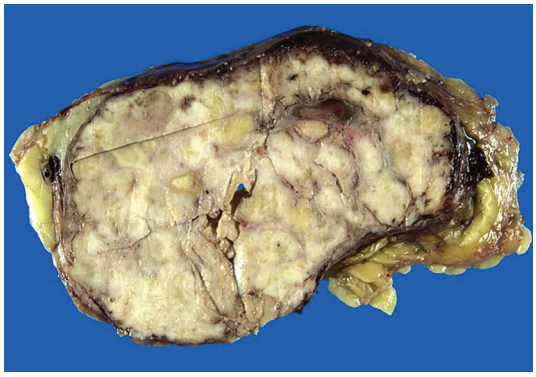

Fig. 16.9: Well-circumscribed tumor with cut surface showing variable smooth muscles, adipose tissue and vessels

- Smooth muscle component appears to originate and radiate from vessel walls.
- They can be arranged in the form of fascicles with cigar-shaped nucleus and abundant eosinophilic cytoplasm. Mitoses are rare.
- Immunohistochemistry, the tumor cells are positive for HMB45, Melan A, SMA and calponin.

EPITHELIOID ANGIOMYOLIPOMA

- It shows the presence of two patterns.
- One is epithelioid cells (at least 80%) ranging from uniform polygonal cells with abundant eosinophilic cytoplasm and intranuclear inclusions.
- These cells show mild nuclear atypia to bizarre multinucleated cells (Fig. 16.10) and epithelioid giant cells.
- Tumor necrosis or hemorrhage is more common.
- The second pattern consists of epithelioid cells plus plump spindle cells which are more uniform with no atypia and mitotic activity and no intranuclear inclusions.

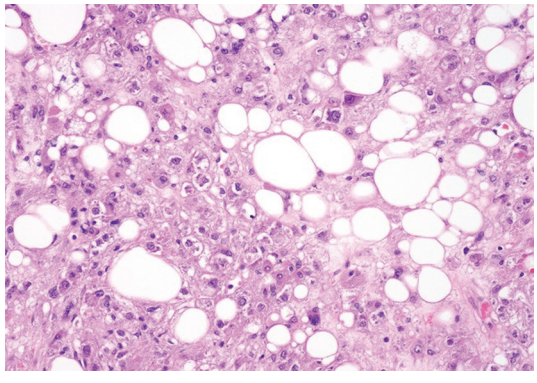

Fig. 16.10: Epithelioid angiomyolipoma showing epithelioid cells along with multinucleate giant cells

- Malignant transformation is seen in high nuclear anaplasia (>70%), geographical necrosis, high atypical mitoses (>2/50 hpf), renal vein and perinephric fat invasion, tumor size >7 cm, old age.

POINTS TO REMEMBER

- Newly recognized epithelial renal tumors are HLRCC-associated renal cell carcinoma, succinate dehydrogenase-deficient renal cell carcinoma, tubulocystic

renal cell carcinoma, acquired cystic disease-associated renal cell carcinoma and clear cell papillary renal cell carcinoma.

- The newly added entities are based on molecular alterations, correlation with specific disease background and familial associated syndromes.
- Paediatric cystic nephroma has been separated from adult cystic nephroma based on DICER1 mutation.
- The identification of the role of immune system in the pathogenesis of clear cell RCC has given way to new targeted therapy.
- Also, immunohistochemistry plays a crucial role to help us distinguish morphologically similar tumors.

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17 Recent Concepts in Autoimmune Hepatitis

INTRODUCTION

- Autoimmune hepatitis (AIH) is a chronic immune-mediated liver disorder characterized by the following features:
  - Female preponderance
  - Elevated transaminase
  - Increased immunoglobulin G levels
  - Seropositivity for autoantibodies
  - Interface hepatitis.
- The disease was first described early in the 1950s, under the name of lupoid hepatitis, as a disease prone to afflicting young women.
- Incidence is highest in North America (42.9/ 100,000), followed by Europe (10.7–23.9/ 100,000).
- In India a few studies on AIH have been published and prevalence has been found to be around 5% of all patients with chronic liver disease.
- There is female predominance with female to male ratio of 4–6:1.
- Although it can affect any age but there is a bimodal distribution with two peaks—
  - First peak around puberty
  - Second peak between 4th and 6th decade.

CLASSIFICATION

- AIH is classified into 3 types according to serum autoantibody.
- Type 1 is most commonly characterized by anti-smooth muscle antibody (SMA) and/ or anti-nuclear antibody (ANA) positivity.
- Type 2, characterized by anti-liver and kidney microsomal antibody (LKM-1) positive, is rare in adults.
- Type 3 is characterized by anti-soluble liver antigen–antibody/anti-hepatopancreatic antibody (SLA/LP) positive.

ETIOPATHOGENESIS

Genetic Susceptibility

- The exact trigger and the underlying mechanism of AIH are still not fully understood.

- The disease has genetically predisposed individuals with susceptible genes, which can be induced by the environment, drugs, infection and other factors.
- HLA is believed to be more closely involved in susceptibility type 1 AIH.
- The immunogenetic background of type 2 AIH has not been extensively studied because of lack of patients.
- The association between the HLA-DR or HLA DRB1 allele and AIH is widely accepted.
- Other studies also show that another locus of HLA (A, B, C, or DQ) may be closely associated with AIH.
- There is a close association between AIH and HLAs in Caucasian patients (HLA-DR3 and DR4) and Japanese patients (HLA-DR4).
- In Pakistan, HLA-DR6, DRB1/14, and DRB1/13 were more prevalent in AIH.
- However, in Latin Americans and Germany, DRB1/1301 is correlated with susceptibility to type 1 AIH.
- HLA phenotype not only indicates the susceptibility of the population to AIH, but also is related to the clinical characteristics of patients.
- Some studies have shown that there is a high prevalence of advanced liver disease in primary diagnosis and a poor outcome of AIH in the patients of India and Pakistan due to the differences in HLA.
- This may indicate that the progression of AIH is different in different races.
- HLA-A1-B8-DR3-positive patients are most commonly characterized by younger ages, heavier condition, worse prognosis, and often need liver transplantation.
- HLA-DR4 positive patients are mostly elder women (50–60 years old), with significantly higher serum gamma globulin, mild condition and will be better after hormone treatment.
- Another study shows that the CTLA-4 +49 A/G polymorphism may be associated with the susceptibility to AIH.

Molecular Mimicry in AIH

- AIH is characterized by a lack of immune tolerance to hepatocyte antigens.
- It eventually leads to liver parenchymal cell damage mediated by autoreactive T cells.
- Immune tolerance refers to a specific unresponsive state when immunologically active cells are exposed to antigenic substances.
- An epitope of liver autoantigen binds with the paratope of an HLA class II antigen.
- It is then exhibited on the surface of antigen-presenting cells.
- Potentially, viral infection or environmental toxin could change hepatocyte epitopes, which could trigger an immune response, possibly via molecular mimicry.
- Molecular modeling hypothesis indicates that pathogens such as bacteria, viruses, drugs and poisons are closely related to the autoimmune response.
- The antigenic epitopes of viruses or exogenous substances are cross-reactive with specific hepatocyte surface antigens.
- However, this mechanism has not yet been confirmed in humans, only proved by mouse model.
- The data available demonstrates that at least in mice, viral infections can break immune tolerance down and lead to autoimmune damage.

Immune Pathogenesis: Cellular Immunity, Humoral Immunity and Cytokines, Treg Cell Deficiency

- **Cellular immune mechanism in AIH:** Cytotoxic T cells (CTL) can be divided into CD4 CTL and CD8 CTL, which can result in liver injury.
- It is generally believed that the molecular mechanism of CTL-mediated target cytotoxicity contains the following two ways:
  - First, the **perforation-based degranulation pathway**, rapidly induces the target cell death, which mainly refers to CD8 CTL.
  - Another pathway is the **Fas-mediated hepatocellular apoptosis**, the Fas ligand (FasL) on the CTL binding with the target cell-associated Fas antigen-induced apoptosis of target cells.
  - This mainly refers to CD4 CTL.
- Immunohistochemical studies showed that Fas was expressed on hepatocytes in patients with AIH.
- Liver histology with AIH showed that portal vein and interval are mainly occupied with CD4 T cells.
- In the liver parenchyma necrotic areas are rich in CD8 T cells.
- Rarely B and NK cells are seen.
- **Humoral immunity with antibody-dependent cell-mediated cytotoxicity (ADCC) in AIH:** In autoimmune liver disease plasma cells secrete a large number of anti-liver cell antigen autoantibodies.
- These have a synergistic effect with T cells.
- They interact with the protein components of the liver cell membrane to form immune complexes.
- Then natural killer cells (NK cells) correspond to the binding of IgG Fc fragment bound to target cells by its surface receptors.
- After that NK cells activate other NK cells to release perforin, granzyme and other cytotoxic substances to kill target cells
- Animal models have shown a large number of IgG on the surface of liver cells, indicating the humoral immunity to liver cell immune injury, and also creating the conditions for ADCC.
- The treatment mechanism of glucocorticoids and azathioprine for autoimmune liver disease is different.
- Glucocorticoid can reduce cytokines, which inhibit the ADCC process of the cell activation, thereby inhibiting the production of CTL and antibodies.
- Azathioprine mainly reduces NK cells selectively to mediate the ADCC process.
- **Immunosuppressive effects of Treg in AIH:** Peripheral tolerance is maintained by CD4+ T cells.
- These cells are characterized by high expression of the interleukin-2 (IL-2) receptor alpha chain (CD25) and low expression of IL-7 receptor (CD127).
- Transcription factor FOXP3+ cells play a crucial role in the pathogenesis of AIH.
- Tregs are essential for maintaining the homeostasis of the immune system and suppressing excessive immune reactions in autoimmune diseases.
- In the AIH patients, the number of Treg cells decreased and the function decreased.
- The lack of Treg cells is the basic cause of AIH.

- Treg cells can express a variety of costimulatory molecules, including CTLA-4, which is a negative regulator of the immune response.
- Treg cells also express liver tissue homing chemokine receptor CXCR3.
- It binds to its ligands CXCL9–11 expressed on inflamed hepatic sinusoid, hepatocytes and bile ducts.
- Recruitment of cells including Treg to the liver is mediated by the tissue homing chemokine receptor, CXCR3.
- It interacts with the IFN inducible chemokines CXCL9–11 on hepatic sinusoids, hepatocytes and biliary epithelial cells.

CLINICAL PRESENTATION

- It varies from being asymptomatic to acute severe or even fulminant hepatic failure.
- Two-thirds patients have ≥1 nonspecific symptoms.
- These symptoms along with their occurrence are shown in Table 17.1.

Table 17.1: Symptoms of AIH and their occurrence

Symptoms	*Occurrence (%)*
Fatigue	86
Jaundice	77
Upper abdominal discomfort	48
Pruritus	36
Myalgias	30
Anorexia	30
Diarrhoea	28
Cushingoid features	19
Fever	18

The physical signs and their occurrence in AIH are presented in Table 17.2.

Signs	*Occurrence (%)*
Hepatomegaly	78
Jaundice	69
Spider angiomata	58
Concurrent immune disease	<38
Splenomegaly	>32
Ascites	20
Encephalopathy	14
None	<25

Microscopy

- Microscopic examination shows features of interface hepatitis.
- The portal inflammatory infiltrate consists primarily of lymphocytes with scattered plasma cells (Fig. 17.1).
- There is also the presence of lobular infiltration (Fig. 17.2).

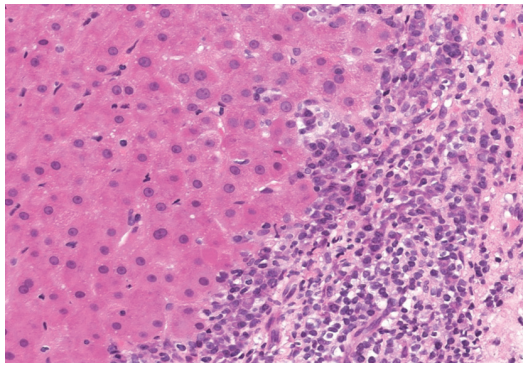

Fig. 17.1: Interface hepatitis with portal inflammatory infiltrate consisting primarily of lymphocytes with scattered plasma cells

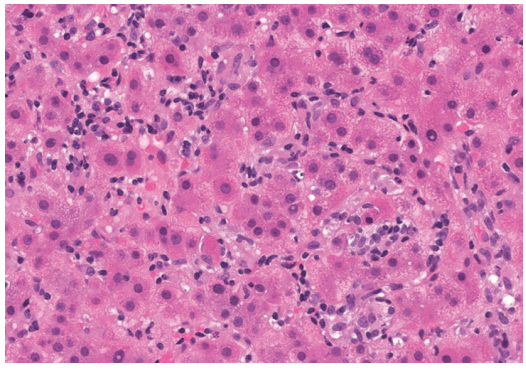

Fig. 17.2: Lobular infiltration

- The major differences in the two major subtypes of AIH, AIH-1 and AIH-2 are shown in Table 17.3.

Table 17.3: Differences between AIH-1 and AIH-2.

	Type 1 AIH	*Type 2 AIH*
Relative prevalence	>80%	20% in Europe, 4% in the USA
Autoantibodies commonly associated	ANA, SMA	LKM1
Patient demographic	Female: male ratio 4:1	Female: male ratio 9:1
Age of onset	Peak incidence 16–30 y, although 50% are >30 y	Average 10 years old but seen in adults (Europe)
Other commonly associated autoimmune diseases	Prevalence of 17–48%: Thyroid disease, rheumatoid arthritis, ulcerative colitis	Prevalence unclear: Type 1 DM, thyroid disease, vitiligo, pernicious anemia, IgA deficiency
Presentation	Acute onset rare	Frequently presents with cirrhosis in children and more aggressively
Response to treatment	Excellent response	May be more treatment resistant
Progression of disease	25% have cirrhosis at diagnosis; 45% develop cirrhosis	~80% develop cirrhosis

DIAGNOSIS OF AIH

- International Autoimmune Hepatitis Group (IAHG) originally proposed criteria for diagnosis of autoimmune hepatitis in 1992.
- These criteria were revised in 1999.
- The revised criteria comprise 12 parameters.
- However, these are too complex for routine use.
- Revised 1999 descriptive criteria for diagnosis of autoimmune hepatitis.

- In 2008, IAHG proposed simplified criteria that incorporate four parameters:
  - Autoantibodies
  - Serum immunoglobulin G
  - Liver biopsy findings
  - Results of viral hepatitis work-up.

Autoantibodies

- The autoantibodies seen in AIH are:
  - Antinuclear antibodies (ANA)
  - Smooth muscle antibodies (anti-SMA)
  - Liver kidney microsome 1 and 2 (anti-LKM 1 and 2)
  - Antibodies to soluble liver antigen (anti-SLA)
  - Antibodies to liver cytosol type 1 (anti-LC1)

Antinuclear Antibodies (ANA)

- These are antibodies that bind to the contents of the cell nucleus.
- They are present in 80% of adults with AIH.
- Indirect immunofluorescence is the reference method for screening and titration.
- Various nuclear patterns seen are homogeneous (most common), speckles, nuclear envelope and nucleolar.

Smooth Muscle Antibodies (anti-SMA)

- They are present in 63% of adults with AIH.
- Concurrent SMA and ANA positivity is the most diagnostic (74% cases).
- However, it is important to remember that anti-SMA is also seen in 3% to 16% of patients with alcoholic liver disease, NAFLD, chronic hepatitis C, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).

Liver Kidney Microsome 1 and 2

- LKM-1: Associated with type 2 AIH and chronic HCV infection.
- LKM-3: Associated with type 2 AIH and chronic HDV infection.
- Anti-LKM-1 has high specificity (99%) but low sensitivity (1%).

Antibodies to Soluble Liver Antigen (anti-SLA)

- It is present in 7–22% AIH.
- Genetic association is seen with HLA DRB1*0301.
- It is useful in diagnosing seronegative patients.
- Specificity is high 99% but sensitivity is quite low at 11%.

Antibodies to Liver Cytosol Type 1 (anti-LC1)

- They are present in 24–32% of anti-LKM1-positive AIH.
- Occur in chronic hepatitis C and anti-LKM1.
- Useful in classifying seronegative AIH.

Liver Biopsy Findings for the Diagnosis of AIH in the 2008 Simplified Criteria

- Typical AIH:
  - Interface hepatitis with lymphocytic or lymphoplasmacytic infiltrates extending into the lobule.
  - Hepatocyte rosette formation.
  - Emperipolesis: Characterized in AIH by the presence of a lymphocyte or rarely a plasma cell within the cytoplasm of hepatocytes. It is found in 65–78% of AIH cases.
- Compatible with AIH:
  - Chronic hepatitis with lymphocytic infiltration without all the features considered typical.
- Atypical–Evidence of another diagnosis.
- IAHG (2008) simplified criteria are displayed in Table 17.4.

Table 17.4: IAHG (2008) criteria for the diagnosis of AIH

Variable	Cutoff	Points
Antinuclear antibody or smooth muscle antibody	Equal to or greater than 1:40	1
Antinuclear antibody or smooth muscle antibody	Equal to or greater than 1:80	2
Or liver–kidney-microsomal antibody	Equal to or greater than 1:40	1
Or soluble liver antigen	Positive	1
Serum immunoglobulin G	More than upper normal limit	1
	More than 1.10 times the ULN	2
Liver histology (evidence of hepatitis is a necessary condition)	Compatible with autoimmune hepatitis	1
	Typical for autoimmune hepatitis	2
Absence of viral hepatitis	Yes	2

The individual scores of antibodies are added up to a maximum of 2 points.

Interpretation

- **≥6 points:** Probable autoimmune hepatitis (88% sensitivity and 97% specificity).
- **≥7 points:** Definite autoimmune hepatitis (81% sensitivity and 99% specificity).

OVERLAP SYNDROMES

- These are certain variant forms of AIH that present with characteristics of AIH and primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC).
- 7% to 13% of patients with AIH have overlapping features of PBC.
- 6% to 11% of patients have features of PSC.
- The features of these syndromes are summarized in Table 17.5.

Table 17.5: Diagnostic features of overlap syndromes

Overlap syndrome	*Serological features*	*Histological features*	*Cholangiographic findings*
AIH-PBC	⅄ AMA positive	⅄ Interface hepatitis ⅄ Destructive cholangitis (florid duct lesions)	⅄ Normal
AIH-PSC	⅄ AMA negative	⅄ Interface hepatitis ⅄ Ductopenia, portal edema or fibrosis ⅄ Obliterative fibrous cholangitis (rare)	⅄ Bile duct strictures

Pathogenic Hypotheses for the Overlap Syndromes of AIH

- They may simply represent a classical disease with variant or atypical manifestations.
- They could represent a transition stage in the evolution of classical PBC or PSC.
- They could represent two diseases in the same individual
- For the diagnosis of overlap syndromes, Paris criteria have been proposed (Table 17.6).

Table 17.6: Paris criteria for diagnosing PBC-AIH syndrome

Primary biliary cirrhosis criteria	*Autoimmune hepatitis criteria*
1. Serum alkaline phosphatase level ≥2x the upper limit of normal (ULN) or serum γ-glutamyltransferase level ≥5x ULN	1. Serum alanine aminotransferase level ≥5x ULN
2. Positive test for antimitochondrial antibodies	2. IgG level ≥2x ULN or a positive test for anti-smooth muscle antibodies
3. Liver biopsy showing florid bile duct lesions	3. Liver biopsy showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis
A diagnosis of PBC-AIH overlap syndrome requires meeting at least two of the three accepted criteria for each condition.	

- The Paris criteria have a sensitivity of 92% and a specificity of 97%.
- They have been endorsed by the European Association for the Study of the Liver (EASL) with the stipulation that all patients have interface hepatitis on histological examination.

DIAGNOSTIC CHALLENGES

Autoantibody-negative Patients

- 10–20% of AIH patients are seronegative for the conventional autoantibodies.
- Antibodies may appear after a few weeks or even after immunosuppressive therapy is begun.
- They may remain negative despite repeat testing.
- Evaluate for the highly specific anti-SLA/LP antibodies, if available.
- If patients meet the diagnosis of AIH based on other criteria, they should be considered for immunosuppressive therapy, regardless of antibody status.

Viral Hepatitis

- 20–40% of patients with chronic HBV or HCV are persistently positive for various autoantibodies.
- However, usually titers are low (1:20 or 1:40).
- Distinction of chronic viral hepatitis from AIH is important.
- It is because IFN therapy can potentially exacerbate autoimmune conditions, and corticosteroids can enhance viral replication.

Hepatocellular Carcinoma and AIH

- HCC occurs in 4% of patients with AIH.
- The risk of HCC is related to:
  - Male sex.
  - Portal hypertension is manifested by ascites, esophageal varices, or thrombocytopenia.
  - Immunosuppressive therapy duration ≥ 3 years.
  - Cirrhosis of at least 10 years duration.
- Therefore, surveillance by abdominal USG should be done every 6 months for these individuals.

TREATMENT OF AUTOIMMUNE HEPATITIS

Absolute Indications for Treatment

- S. transaminases ≥ 10 fold upper limit of normal (ULN) or
- S. transaminases ≥ 5 fold ULN and γ-globulin level ≥ 2 fold ULN
- Histological evidence of necrosis or
- Incapacitating symptoms or acute severe onset.

Relative Indications for Treatment

- Serum transaminases < 10 fold ULN and γ-globulin level < 2 fold ULN.
- Interface hepatitis.
- Mild or no symptoms.

Treatment Regimens

- There are three treatment strategies:
  - Prednisone monotherapy,
  - Initial monotherapy with steroid with subsequent addition of azathioprine,
  - Combination therapy from the start.

Remission

- Ideal endpoint: No symptoms, normal liver tests, normal liver tissue, occurs only in 40%.
- Satisfactory endpoint: No symptoms, AST/ALT ≤ 2 fold ULN, other tests normal, no interface hepatitis.
- Histological remission lags behind clinical and biochemical remission by 3–6 months, explaining prolonged maintenance therapy to accommodate this lag.

Outcome

- Relapse occurs in 50% of cases within 6 months and in 70–86% within 3 years.
- Sustained remission occurs only in 21%.

Newer Drugs

- ***Calcineurin inhibitors.***
- These are used primarily as salvage therapies for steroid-refractory disease.

AIH and Liver Transplantation

- **Indications of liver transplantation in AIH:**
  - Acute liver failure.
  - Decompensated cirrhosis
  - Hepatocellular carcinoma meeting transplant criteria (uncommon).

Recurrent AIH in Allografts

- Recurrence occurs in 12–46% of patients.
- The incidence increases with time after liver transplantation and accelerates after discontinuation of steroids.

POINTS TO REMEMBER

- Autoimmune hepatitis (AIH) is a condition that is often underestimated or unrecognized due to the large heterogeneity of the genetic, clinical, laboratory, histological and serological features.
- The International Autoimmune Hepatitis Group (IAHG) has published detailed guidelines on how to test for autoantibodies relevant to AIH.
- Liver histology is mandatory for AIH diagnosis.
- The latest IAHG (2008) simplified score takes into account only four parameters and is far easier for everyday use in clinical practice.
- Treatment is mandatory and usually life-saving in patients who have clinical, laboratory, or histological features of active liver inflammation.
- Alternative therapies, such as ciclosporin, tacrolimus, or other new drugs, including biological agents, are very promising.
- A network of committed clinical investigators must be generated to evaluate new therapies in multicenter studies.

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Advances in the Pathology of Glomerular Diseases

18

Anisha Manocha

INTRODUCTION

- Glomerular diseases are classified as:
  - Primary glomerulopathies
  - Systemic disease with glomerular involvement
  - Hereditary disorder
- The renal disease patterns are based on four morphological components.
- These four components are interdependent and disease affecting one may affect the other components secondarily in the course of time.
  1. **Glomeruli**
     - Diseases affecting glomeruli present with hematuria, proteinuria, oliguria, puffiness of the face, generalized edema and azotemia.
     - Usually immune-mediated.
  2. **Tubules**
     - These present with inability to concentrate urine polyuria, nocturia, electrolyte disturbances, acute renal failure and azotemia.
     - Usually due to toxins/infections/ inherited.
  3. **Interstitium**
     - Interstitial diseases present with inability to concentrate urine, acute renal failure and pyuria.
     - Usually due to drugs/infections.
  4. **Blood vessels**
     - They present with hypertension, acute renal failure and ischemic injury leading to papillary necrosis/infarcts.
     - Usually congenital or due to drugs.

Glomerular Syndromes

These include the following:

1. **Nephritic syndrome:** Hematuria, azotemia, mild proteinuria and hypertension.
2. **Nephrotic syndrome:** >3.5 gm/day protein excretion in urine, hypoalbuminemia, hyperlipidemia and lipiduria.
3. **Rapidly progressive glomerulonephritis:** Proteinuria, hematuria and acute renal failure.

4. **Chronic renal failure:** Azotemia leading to uremia.
5. **Isolated urinary abnormalities:** Hematuria or proteinuria.

Advances in Glomerular Pathology

- Glomerular injury can be caused by numerous insults:
  - Hemodynamic
  - Infection
  - Immunological
  - Hereditary
  - Metabolic diseases
  - Toxicity.
- The understanding of glomerular pathophysiology and pathology have been advanced considerably by the combination of clinical and pathological observations regarding:
  - Patterns of injury
  - Clinical syndromes.
- Renal biopsy allowed us to observe the patterns of renal disease by microscopic analysis, including electron microscopy and immunofluorescence.
- These new tools have combined with major advances in the understanding of molecular and cellular basis of the glomerular microcirculation, its permselectivity and tubular functions.
- Based on compartmentalization of the glomerulus, four patterns of glomerular injury are recognized:
  - Epithelial
  - Endothelial
  - Mesangial injury
  - Chronic glomerulosclerosis.
- These four patterns encompass most glomerular diseases and correlate with four distinct clinical syndromes as follows:
  - Nephrotic syndrome
  - Nephritic syndrome
  - Hematuria
  - Asymptomatic proteinuria.

Epithelial Cell Injury–Podocytopathies and Membranous Nephropathy

- Newer developments in the study of glomerular visceral epithelial cell (GVEC) injury have contributed to:
  - Better understanding of minimal change nephropathy (MCNS)
  - Knowledge about various forms of focal and segmental glomerulosclerosis (FSGS).
- Injury to the podocyte leads to the following changes:
  - Loss of interdigitating foot processes.
  - Detachment from the underlying basement membrane.
  - Disruption of the glomerular sieve.
  - Change in charge—selective barrier resulting in massive proteinuria.

Minimal Change Nephropathy (MCNS)

- In this case, the injury can only be observed by electron microscopy and is usually sensitive to steroid treatment.

- It is the most frequent form of nephrotic syndrome in children but can also occur in adults.
- Clinical and experimental studies have provided the following information:
  - It is an immune-mediated disease.
  - Podocytes are affected by circulating cytokines.
  - It may or may not be associated with hypersensitivity reactions and malignancies.
  - However, the molecular basis for MCNS is still uncertain.

Focal Segmental Glomerulosclerosis (FSGS)

- The idiopathic form of **focal segmental glomerulosclerosis (FSGS)** is considered to be part of the same spectrum as MCNS.
- That is why often, in the early phase it is sensitive to steroid treatment.
- In many cases, FSGS shows a relapsing pattern of nephrotic syndrome.
- This ultimately leads to an unresponsive form of glomerular injury and to end-stage renal failure.
- Originally, FSGS was a pattern of injury described in a wide variety of renal diseases.
- In the last 10 years, this descriptive diagnosis has been challenged and classified based on disruption of podocyte genes encoding cell-adhesion and cell-signaling proteins.
- Modifying genes such as APOL1 may affect cellular injury of podocytes under stress, facilitating the development of FSGS
  - Two groups of podocytopathies have been identified as causes of FSGS:
    - Gene disruption involving nephrin, podocin, CD2AP, α-actinin, integrin α3βα1, CD151 tetraspanin, TRPC6, laminin B2, collagen XVII, Ilk, Rhoa, Myo1E, IFN2, MYH9, dystroglycan and heparan sulfate moieties.
    - Extrinsic factors including intracapillary hypertension, viral infection, toxicity, complement activation, metabolic storage diseases.
  - The identification of the different causes of podocytopathies has led to:
    - Facilitation of the management of patients with secondary forms of FSGS
    - Improvement in their prognostic evaluation, especially the prospect of transplantation.
  - Studies on the biology of glomerular visceral epithelial cell (GVECs) have revealed the following:
    - Pathophysiology of cell–cell and cell–matrix interaction
    - Factors governing the integrity of the cell and the glomerular capillary filter
    - Mechanosensing
    - Heparan sulfate modulation
    - Proton pump function
    - Cathepsin L activation
  - Soluble urokinase plasminogen activator receptor (suPAR) as a permeability factor has been proposed in FSGS.
  - However, different studies have yielded conflicting results.
  - Thus, at present the role of suPAR in FSGS remains unclear.

Membranous Glomerulopathy

- It is another of nephrotic syndrome due to GVEC injury.
- MN is an immune-mediated glomerular injury.
- Immune complexes of immunoglobulins and complement accumulate in a granular pattern along the outer side of the GBM leading to GVEC detachment.

- The immunoglobulins can be directed against autoantigens, produced by the GVEC or extrinsic antigens accumulating at the specific site due to charge interactions.
- The podocyte is injured by the local formation of complement stimulated by the nearby immune complexes.
- In secondary MN, it is hypothesized that positively charged viral proteins or nuclear histones may cause immune deposit formation on the subepithelial side of the GBM.
- Bovine serum albumin is the first dietary antigen to be unequivocally involved in immune-mediated glomerulopathies.
- Thus, there is a role for absorbed dietary antigens and more generally for environmental factors in the pathogenesis of glomerular diseases.
- Idiopathic autoimmune MN:
  - Autoantibodies are of the IgG4 and IgG1 type.
  - They are directed against the phospholipase A2 receptor (**PLA2-R**).
  - These antibodies affect the GVEC directly through interaction with PLA2-R and can bind complement components.
  - Formation of the membrane attack complex leads to cell injury and death.
  - Distinction between idiopathic and secondary forms of MN has been facilitated by the introduction of assays to determine PLA2-R autoantibodies in serum and PLA2-R antigen in renal biopsies.
  - Further work is needed to establish whether circulating levels of anti-PLA2R1 antibodies can predict disease outcome or recurrence in the grafted kidney after transplantation.
  - Genome-wide association studies have shown:
    - Autoimmune response in patients with idiopathic MN to be based on two single nucleotide polymorphisms
    - One for the gene encoding PLA2-R, and other for the HLA antigen-presenting peptide within the HLA-DQA2 complex.
  - Further insight into the process underlying PLA2-R self-recognition may facilitate the design of epitope-blocking reagents.
  - Anti-CD20 monoclonal antibody rituximab has been shown to be a promising therapy for idiopathic MN.
  - This represents a paradigm shift in treatment, allowing fresh insights in pathophysiology.
  - Furthermore, treatment can be aimed at the level of tissue injury in particular targeting complement component activation and regulation.
  - Other antibodies directed against enolase, superoxide dismutase 2, and aldose reductase have also been identified in the serum but their role in the initial development of MN remains controversial.

Endothelial Cell Injury–ANCA-Associated Renal Pathology

- Numerous large studies carried out on a large cohort of patients have shown highly restricted polymorphisms in genes encoding HLA-DP, proteinase 3 and SERPINA1 in patients with ANCA-associated systemic vasculitis and glomerulonephritis.
- In this disease, the endothelial compartment is injured leading to the following features:
  - Fibrinoid necrosis
  - Capillary wall destruction
  - Extracapillary proliferation
  - All the above three cause permanent damage to the glomerular tuft.

- The understanding of etiopathogenesis has been greatly aided by the following major discoveries:
  - Identification of proteinase 3 and myeloperoxidase as targets for ANCA.
  - Pathogenesis of endothelial injury and vasculitis due to a direct interaction of ANCA with neutrophils and endothelium.
  - Role of infection with *Staphylococcus aureus.*
  - Relevance of prevention by antibiotic treatment.
- New advancements will try to target epitope-guided treatment so as to modify the genetic response in these cases.
- The role of complement in the ANCA-associated endothelial injury is less well understood.
- Immunofluorescence shows only segmental low-level deposition of immunoglobulins and complement components.
- This has led to many authors designating it as *pauci-immune glomerulonephritis*.

Complement System and Glomerular Pathology

- The components of the complement system have an important role in opsonization and removal of microbial antigens, dead cells and tissue fragments.
- Complement pathway can be activated by the following:
  - Antibodies
  - Serum proteins of the collectin family
  - Microbial surface glycoproteins
  - Extracellular matrix components
- Based on these three routes of activation are there:
  - Classical.
  - Mannan-binding lectin.
  - Alternative pathways.
- The complement system can induce the removal of debris in a quiescent way through digestion by macrophages and B cells with induction of immune tolerance.
- However, in cases of massive or persistent debris the system may initiate the inflammatory pathway by recruitment of phagocytes through chemotaxis leading to tissue inflammation and breakdown followed by immunization.
- The components of the complement system are held in check by a large number of regulatory and inhibitory proteins in the circulation and at the surface of endothelial cells.
- Recent studies have found that dysregulation of the complement system may cause induction of systemic lupus erythematosus.
- This happens by inadequate removal of apoptotic nuclear fragments leading to autoimmunity to DNA and phospholipids.

Endothelial Cell Injury: Complement and HUS

- Inappropriate activation of the complement system due to ineffective inhibition may lead to prolonged tissue damage.
- This mechanism has recently been reported to underlie the *Escherichia coli-associated and atypical forms of hemolytic uremic syndrome* (aHUS)
- It is also involved in the two idiopathic forms of membranoproliferative glomerulonephritis (MPGN) previously known as type I and type II MPGN.

- Using immunofluorescence some authors have reported cases of MPGN with isolated C3 deposits.
- These are characterized by defective control of the alternative pathway of complement.
- They have been designated as C3 glomerulopathies.
- On the other hand, MPGN type I shows deposits of immunoglobulin and complement.
- These occur due to activation of the classical pathway by antigen–antibody immune complexes.
- However, this distinction is not agreed upon by other workers.
- It has been found in some studies that alternative pathway is involved in both of these types.
- Sixty percent of aHUS cases are related to the following gene mutations:
  - Complement regulatory proteins, factor H (CFH)
  - Membrane-cofactor protein (MCP)
  - Factor I (CFI)
  - Thrombomodulin (THBD)
  - Alternative pathway C3 convertase components C3 and factor B (FB)
  - Inhibitory anti-CFH autoantibodies.
- Cases with combined mutations in two or three different complement genes have also been reported.
- C3NeF, an autoantibody stabilizing the AP C3 convertase, is found in 50–75% of patients with C3 glomerulopathies but also in about half of patients with MPGN I.
- Autoantibodies against CFH and factor B and complement gene mutations have also been reported.
- For C3 glomerulopathies, there is no specific treatment.
- Eculizumab (anti-C5 antibody) reduced proteinuria in a few patients but others did not respond.

Renal Pathology and the Prognosis of Glomerular Disease

- In all major glomerulonephritis strong correlation of chronic glomerular injury (adhesions, scars) with poor outcome has been observed.
- Molecular and proteomic studies on renal tissue and urine have been initiated.
- The aim of these studies is to correlate transcription and peptide profiles with outcomes.
- This will allow a more precise intervention in signaling pathways involved in inflammation and repair.

POINTS TO REMEMBER

- Glomerular diseases are a major cause of renal disease.
- Over the last century major advancement has occurred in knowledge and understanding of these diseases.
- However, still a lot remains to be learned.
- To this end many basic and translational experimental studies in combination with clinical research have been carried out.
- This new knowledge has facilitated classification and treatment and has contributed to a better outcome in these patients.
- Since renal disease also often lead to systemic especially cardiovascular complications, these advances are also of general health interest.

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19 Autoimmune and Drug-Induced Lymphadenopathies

Harsh Batra

INTRODUCTION

- Lymphatics is a network of organs, lymph nodes, lymph ducts and lymph vessels that make and drain lymph from tissues to bloodstream.
- The lymphoid tissue is concerned with immune function in defending body against foreign antigens.
- The finding of lymphadenopathy usually raises concern about an underlying illness and mostly it is a result of a benign infectious cause.
- The body has approximately 600 lymph nodes.
- But only those in the submandibular, axillary, or inguinal regions may normally be palpable in healthy people.
- Lymphadenopathy refers to nodes that are abnormal in either size, consistency, or number.

Types of Lymphoid Organs

- These are of two types:
  - *Primary lymphoid organs:* Thymus and bone marrow.
  - *Secondary lymphoid organs:* Lymph node, tonsil and others.
- Lymphoid tissue enlarges until puberty and progressively atrophies throughout life.

Functions of Lymphoid Organs

- Removal of interstitial fluid from tissues that is collection of lymph plasma.
- Absorption and transport of fatty acids and fats.
- Defense mechanism for the body.

AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS)

- This is a disease that arises from a disruption in the immune system secondary to a defective mechanism of FAS-mediated apoptosis.
- It is a rare disorder with an autosomal dominant pattern of inheritance.
- Apoptotic defects lead to a lymphoproliferative disease with clinical manifestations including:
  - Lymphadenopathy
  - Hepatomegaly

- Splenomegaly
- Autoimmune disease
- Secondary malignancies.

- The two most common mutations involve FAS mutation (65%) and CASP10 (25%).
- Most patients present with symptoms within one year.
- **Pathophysiology:** Normal control of lymphocyte proliferation and immune tolerance is essential for both host defense and protection against self-directed immune attacks.
- This process is most often mediated by the cell surface receptor FAS, a member of the tumor necrosis factor receptor (TNFR) superfamily, also termed CD95/APO1.
- FAS maintains lymphocyte homeostasis and peripheral immune tolerance to prevent autoimmunity.
- A mutation causes double-negative T cells to proliferate.
- Although critical in pathogenesis other factors also play a role.
- Modifier genes as well as environmental factors may be involved.
- Furthermore, the clinical penetrance of heterozygous *FAS* mutations (described as 70% in the presence of one mutation) suggests that a "second hit" may be required for disease onset.
- **Microscopy:**
  - *Marked paracortical expansion of T cells:* Lymphocytes at different stages of immunoblastic transformation (small, intermediate and large cells).
  - Increased numbers of immunoblasts and frequent mitotic figures, scattered plasma cells in the background
  - Germinal centers show reactive changes that vary from florid follicular hyperplasia, progressive transformation of germinal centers to atrophic follicles.
- **Immunohistochemistry:**
  - Markedly increased number of double negative T cells (CD45RA+, CD45RO–, CD3+, CD4–, CD8–)
  - Increased number of CD57+ and perforin+ T cells.
  - No clonal rearrangements of T or B cell genes are present.
- **Differential diagnosis:**
  - Thymoma
    - Most thymomas contain epithelial cells that are positive for CK.
    - This is not seen in ALPS.
  - T cell lymphoma
    - The lymph node architecture is effaced in lymphoma.
    - Additionally, T cell lymphomas will typically show loss of common T cell antigens (CD2, CD5, CD7).
    - Peripheral T cell lymphomas are very uncommon in the pediatric setting, with the exception of ALK+ anaplastic large-cell lymphoma.
    - ALCL would not be confused with ALPS in morphology.
  - Classic Hodgkin lymphoma:
    - It has the presence of the characteristic Reed-Sternberg cells and variants that are CD30+, CD15+, PAX5+ (dim) and CD45–.
    - The reactive immunoblasts of ALPS are CD15–, PAX5–, and CD45+.
  - Viral lymphadenitis:
    - Dual CD4–/CD8– T cells are not seen in viral infection.

SARCOIDOSIS

- It is a multisystem disorder of unknown etiology.
- It is characterized by the formation of immune granulomas in various organs, mainly the lungs and the lymphatic system
- Sarcoidosis affects people of all ages regardless of race and ethnicity, with peak incidence among those aged 20 to 39 years.
- It is thought to be more common in rural communities.
- **Clinical features:**
  - Constitutional symptoms include fatigue, malaise, weight loss and night sweats.
  - Organ involvement includes multisystem involvement which includes lungs (dyspnea, cough); eyes (keratoconjunctivitis, uveitis, retinal vasculitis), skin (rashes, erythema nodosum) and musculoskeletal system involvement (arthritis).
- When LN involvement is present, non-necrotizing and necrotizing granulomas can be present.
- **Pathogenesis:**
  - *Genetic factors:*
    - There is familial clustering in monozygotic twins.
    - Afro-American population shows greater susceptibility.
    - Polymorphism in butyrophilin-like 2 receptor gene.
  - Mycobacteria and propionibacterium involvement has been suggested by some authors.
  - *Immunological factors:* Initially, an antigenic peptide is presented to the T cell receptor on naive T cells via human leucocyte antigen class II molecules on antigen-presenting cells.
  - This results in activation with subsequent clonal proliferation and T helper (Th1) polarization with release of cytokines and chemokines.
  - Th1 cells and macrophages stimulate monocytes to form non-caseating granulomas which may persist or regress.
  - Fibrosis is an alternative outcome through a transition from a Th1 to a Th2 cytokine profile.
  - Th1 cytokines are important in promoting granulomatous inflammation and inhibiting fibrosis.
- **Lab diagnosis:**
  - Hypercalcemia
  - Hypercalciuria
  - Elevated serum ACE (75% cases).
  - Bronchioalveolar lavage: Lymphocytic pleocytosis, characterized by an increase in CD4: CD8 ratio on flow cytometry.
  - Bilateral hilar lymphadenopathy on CT scans with associated reticular opacities.
- **Microscopy:**
  - The granulomas are composed of epithelioid histiocytes with scattered **Langhans type giant cells** and small lymphocytes.
  - Absence of necrosis is the classic finding, however, small central foci of fibrinoid necrosis can be present in approximately 10% of cases.
  - The characteristic findings are:
    - Schaumann bodies (round with concentric laminations of calcium).
    - Asteroid bodies (star-like structures with calcium).

- Hamazaki-Weisenberg inclusions (yellow-brown, ovoid, large lysosomes with hemosiderin).
- It is to be noted that these are not specific for sarcoidosis.

- **Differential diagnosis:**
  - Infectious lymphadenitis, particularly from fungal or mycobacterial organisms:
    - Fungal (PAS, GMS) and mycobacterial (AFB, FITE) stains can help distinguish.
    - Immunostains for mycobacterial organisms are also available.
    - There is often the presence of suppurative granulomas with necrosis.
    - Typically, not seen in sarcoidosis.
  - Chronic granulomatous disease (CGD)
    - It is a disease of children.
    - There are recurrent bacterial and fungal infections
    - The underlying defect is mutations in various genes that encode the subunits of the superoxide-generating phagocyte NADPH oxidase system.
    - Various organs and systems can be affected including the lymph nodes.
    - LN shows non-necrotizing and necrotizing granulomas.
    - The diagnosis is made on the basis of the abnormal nitroblue tetrazolium (NBT) reduction test.

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

- It is an autoimmune disorder with frequent involvement of lymph nodes and spleen.
- **Clinical features:**
  - Malar rash
  - Photosensitivity
  - Ulcers in the mucous membranes.
  - Arthritis
  - Serositis.
- LN involvement is present in 12–78% of patients with SLE.
- ***Cervical, inguinal and axillary*** are most commonly involved.
- **Lab diagnosis:**
  - Detection of autoantibodies including ANA, anti-dsDNA and anti-SM antibody. etc.
- **Lymph node microscopy:**
  - Follicular hyperplasia, necrosis (sometimes large, geographic areas).
  - There is proliferation of various cells including macrophages, immunoblasts and plasma cells.
  - Areas of necrosis show ***neutrophils*** and ***neutrophilic debris***.
  - Interfollicular polyclonal ***plasmacytosis*** can be seen, in addition to the presence of plasma cells within the follicles.
  - Hematoxylin bodies are a rare finding (amorphous aggregates of basophilic material, PAS+) in the sinuses, paracortex, or vessel walls.
  - They are diagnostic of SLE.
  - Other patterns include:
    - Castleman-like changes.
    - Follicular hyperplasia with vascular proliferation.
    - Follicle-lysis with mantle cells intruding on the germinal centers.
    - Proliferations of follicular dendritic cells.

- **Differential diagnosis:** Angioimmunoblastic T cell lymphoma or polymorphous post-transplant lymphoproliferative disorders.

KIKUCHI FUJIMOTO DISEASE (KFD)

- It is necrotizing lymphadenitis without neutrophilic infiltration (in contrast to SLE lymphadenopathy).
- Generally seen in people of Asian descent with the age group less than 30 years old.
- Male:female ratio is 1:4.
- Cervical lymph nodes are commonly involved.
- **Clinical features:**
  - Fever
  - Upper respiratory symptoms
  - Very tender lymphadenopathy
- Spontaneous resolution of the symptoms and lymphadenopathy occurs within 1–4 months of the diagnosis.
- **Lab diagnosis:**
  - Neutropenia and lymphocytosis in the peripheral blood.
  - Negative for ANA, dsDNA, and EBV.
- **Lymph node microscopy:**
  - Effacement of the architecture, sometimes patchy in the early phases.
  - It begins in the paracortex and subcapsular region with necrosis and apoptosis of the cells without neutrophilic infiltration.
  - Plasma cells are typically absent or rare.
  - Immunoblasts are numerous in viable areas of the paracortex contiguous with necrosis.
  - Three histologic variants have been described:
    - Lymphohistiocytic or proliferative stage (early)
    - Necrotic
    - Phagocytic/foamy cell (late).
- **Immunohistochemistry:**
  - Large numbers of **CD123+ plasmacytoid dendritic cells** can be an indication for the diagnosis of Kikuchi Fujimoto disease.
  - Histiocytic infiltrates are positive for CD4, CD68, and lysozyme. Most of the T cells are CD8+.
  - The immunoblasts in KFD are CD3+, CD8+, CD30+ T cells.
- **Differential diagnosis:**
  1. Herpes simplex-associated lymphadenitis (HSV-LAD)
     - In contrast to KFD, in HSV-LAD there are multinucleated cells and viral cytopathic changes.
     - Frequent neutrophils are also seen.
  2. *Infections (fungal, mycobacterial):* These can be distinguished with the routine use of special stains (GMS, AFB, FITE, PAS).
  3. *Cat-scratch disease (CSD):*
     - It typically shows stellate-shaped necrosis with abundant neutrophils.
     - Presence of the *Bartonella henselae* can be demonstrated by a Warthin-Starry stain.

RHEUMATOID ARTHRITIS (RA) AND JUVENILE RHEUMATOID ARTHRITIS (STILL DISEASE)

- Lymphadenopathy presents in approximately 75% of patients with RA.
- The commonly involved groups of lymph nodes are cervical, supraclavicular and axillary.
- **Clinical features:**
  - Non-specific symptoms (fatigue, anorexia, fever, musculoskeletal pain).
  - Symmetric joint involvement affecting the hands and feet is typical.
- Adult-onset juvenile RA (Still disease) is characterized by fevers, arthralgias, arthritis, polyserositis, hepatosplenomegaly and lymphadenopathy.
- Lab findings include leukocytosis, hypergammaglobulinemia and negative RF.
- Most cases have very high ferritin levels.
- Lymph node involvement occurs in 40–90% of patients.
- **Lab diagnosis:**
  - *Rose-Waaler test:* The **rheumatoid factor (RF)** is the characteristic serologic marker present in patients with RA.
  - *Synovial fluid analysis:* It is sterile, and shows pleocytosis (usually neutrophils), increased protein, decreased viscosity and decreased complement.
  - *C-reactive protein is elevated.*
- **Lymph node microscopy:**
  - Marked follicular hyperplasia in the cortex and medulla of the lymph nodes.
  - Follicles of various sizes and shapes are present.
  - Germinal centers have a starry-sky pattern.
  - In some cases, hyaline-like eosinophilic deposits in the germinal centers are noted (PAS+, Congo red-).
  - These may show dystrophic calcification.
  - The interfollicular areas show an increased number of plasma cells and capillary endothelial hyperplasia.
  - Neutrophils in the sinuses and interfollicular areas are also seen.
  - Sarcoid-like granulomas have been reported.
  - After immunosuppressive therapies, the follicular hyperplasia is reduced.
  - The interfollicular and paracortical areas become hyperplastic.
  - Patients treated with gold therapy can show non-birefringent crystalline structures throughout the parenchyma.
  - The germinal centers show a normal phenotype (CD10+, BCL-6+, BCL-2-).
  - Follicular dendritic networks are preserved.
  - *In situ* expression of EBV (e.g. EBER) has been reported in 20% of RA cases.
- **Histologic findings in Still disease:**
  - Similar to RA.
  - Lymph nodes show florid follicular hyperplasia, exuberant immunoblastic proliferations, atypical paracortical hyperplasia, and burned-out histiocytic pattern.
  - The paracortical pattern shows a markedly increased density of high endothelial venules.
  - This pattern can mimic angioimmunoblastic T cell lymphoma.
  - However, the hyperproliferation of follicular dendritic networks is absent.
  - Hyperplastic and atrophic germinal centers are also present.

- The interfollicular cells include lymphocytes, immunoblasts, plasma cells, macrophages and rare eosinophils.
- The burned-out histiocytic pattern shows vascular proliferation, numerous macrophages in the sinuses, and effacement of the normal follicular architecture.

- Differential diagnosis includes a T cell lymphoma, SLE, drug-induced lymphadenopathies, and other autoimmune processes.

VASCULITIDES

- These disorders can sometimes show involvement of the lymph nodes and spleen.
- ANCA are important serologic tests for the evaluation of vasculitis.
- ANCA antibodies are frequently encountered in granulomatosis with polyangiitis (GPA) and Churg-Strauss disease.
- **Lymph node microscopy:**
  - LN and spleen in granulomatous polyangiitis show capsular adhesions, vascular thrombosis and infarction.
  - Granulomas with necrosis are also present.
  - The areas of necrosis can be small or large, and are associated with vasculitis, and inflammation of the capsule.
  - In Churg-Strauss disease, there are large, eosinophilic-rich granulomas with focal of central eosinophilic abscesses surrounded by a macrophagic reaction.
  - In HSP, there is sinus histiocytosis, regressed follicles, and replacement of the follicles by granulomas.
  - Germinal centers can show neutrophils and microabscesses.
  - Fibrin deposition in the vessel walls and other features of vasculitis can also be evident.

KAWASAKI DISEASE

- Kawasaki disease (KD) is an acute febrile exanthematous disease of children.
- Vasculitis of **small and medium-**sized arteries throughout the body occurs.
- It is a leading cause of ischemic heart disease in children.
- Approximately 70% patients during the acute phase show cervical lymphadenopathy.
- Besides cervical inguinal and axillary can be infrequently involved too.
- **Microscopy:**
  - Presence of necrosis is seen in 22% of cases.
  - The necrosis starts below the capsule, and ranges in size.
  - Adjacent vessels show thrombosis and nuclear debris.
  - Necrosis with significant inflammation is often present in the capsule and the fibroconnective tissue surrounding the capsule.
  - Inflammatory cells consist mainly of lymphocytes, plasma cells, and monocytes/macrophages, with a ***relative paucity of neutrophils.***
  - Other histologic findings include paracortical zone hyperplasia, sinusoidal expansion and follicular hyperplasia.
- **Differential diagnosis:**
  - Kikuchi Fujimoto disease (KFD)
    - It shows necrotizing lymphadenitis with histiocytes.
    - Expansion of plasmacytoid dendritic cells by CD123 can be helpful.

- There is a lack of neutrophilic inflammatory infiltrate and the presence of vasculitis.

♦ SLE:
- Vasculitis can sometimes be present.
- Hematoxylin bodies are classic of SLE.
- Acute inflammation can also be seen in SLE.

♦ *Kimura disease:* As described below.

KIMURA DISEASE (KD)

- Synonyms include eosinophilic lymphogranuloma and eosinophilic lymphoid follicular hyperplasia.
- It is a rare chronic inflammatory disorder of unknown etiology.
- Commonly presents in the head and neck region, and involves subcutaneous tissues and lymph nodes.
- Major salivary glands can also be affected (parotid and submandibular glands).
- Currently Kimura disease and angiolymphoid hyperplasia with eosinophilia are considered as distinct entities.
- KD has a peak age of presentation in males within the 3rd decade of life.
- It has a particular predilection for individuals of Asian descent.
- Patients present with localized swelling in the head and neck, particularly the preauricular region.
- Systemic symptoms are not frequent.
- However, nephrotic syndrome occurs in 60% of cases.
- Laboratory workup shows blood eosinophilia, and elevated IgE levels.
- **Microscopy:**
  - ♦ Preserved but distorted overall architecture.
  - ♦ Hyperplastic follicles with well-formed germinal centers are present.
  - ♦ Deposition of IgE in the germinal centers forms hyaline proteinaceous material.
  - ♦ A marked eosinophilia is present with the formation of eosinophilic microabscesses and eosinophilic follicle lysis.
  - ♦ A distinctive finding is polykaryotic dendritic cells, most often within germinal centers, but occasionally in interfollicular areas.
  - ♦ Necrosis can be present in some cases.
  - ♦ There is a vascular proliferation in the interfollicular areas with endothelial lining cells displaying cytoplasmic vacuoles.
  - ♦ There is also, stromal and perivascular fibrosis.
  - ♦ Genetic testing shows germline IGH and TCR receptors.
- **Differential diagnosis:**
  - ♦ Angiolymphoid hyperplasia with eosinophilia (ALHE)
    - In contrast to Kimura disease, ALHE is a disease that involves the skin, particularly in the superficial dermis.
    - It has a lobular growth of capillaries or vessels and shows the presence of prominent epithelioid endothelial cells.
    - It does not involve the lymph nodes.
  - ♦ Dermatopathic lymphadenitis:
    - It is characterized by the presence of deposits of melanin or hemosiderin in subcapsular or paracortical histiocytes.

- An increased number of interdigitating dendritic cells and Langerhans cells is seen.
- Dermatopathic changes classically occur in relation to a skin rash, where the regional lymph nodes in proximity to the skin lesions become affected.

- Langerhans cell histiocytosis:
  - It often has a sinusoidal or sinusoidal/paracortical pattern of involvement.
  - Necrosis and eosinophilic microabscesses are common.
  - The Langerhans cells are positive for S100, CD1a and Langerin.
  - They also show BRAF V600E mutations in 50% of cases.

LYMPHADENOPATHY SECONDARY TO MEDICATIONS

- Drug-induced hypersensitivity syndrome (DIHS) is an idiosyncratic host reaction to a medication.
- It involves T cell and macrophage activation which results in a cytokine release.
- The criteria for DIHS includes the following:
  - Causal establishment of the reaction to exposure or re-exposure to a specific medication.
  - Presence of eosinophilia and/or atypical lymphocytosis in the peripheral blood.
  - Involvement of at least 2 organ systems (including the skin).
- The pathogenesis is poorly understood.
- Onset does not correlate with the drug dosage or serum levels.
- The clinical symptoms and lymphadenopathy resolve upon discontinuation of the medication.
- Drugs associated with DIHS:
  - **Antiepileptics** (phenytoin, carbamazepine, phenobarbital, primidone, lamotrigine, gabapentin, and ethosuximide).
  - Antibiotics (minocycline, β-lactams, sulfas);
  - Allopurinol
  - Dapsone.
- Usually cervical and axillary group of lymph nodes is involved.
- **Clinical features:**
  - Fever with skin rash at the onset.
  - Associated with erythema in the oropharyngeal mucosa.
  - Hepatitis, pneumonitis, interstitial nephritis, or arthritis is seen in 20% of cases.
- **Lab findings:**
  - Leukocytosis or leukopenia
  - Atypical lymphocytes in the peripheral blood
  - Eosinophilia
  - Hemolytic anemia.
- **Two main histopathologic patterns** are encountered in the affected lymph nodes by DIHS:
  1. *Acute onset:*
     - Lymph node is enlarged with preserved architecture.
     - There is paracortical expansion of T cells including the presence of large immunoblasts and eosinophils
     - Reed-Sternberg-like cells can also be present.

- The lymphocytic population can extend beyond the capsule into the surrounding adipose tissue.
- A vascular proliferation is common, in addition to the presence of vasculitis.
- Foci of necrosis is also encountered infrequently.
- Follicular hyperplasia is also typical, but less prominent than the paracortical changes.

2. *Late onset DIHS:*
   - Paracortical hyperplasia with fewer eosinophils.
   - Immunoblasts can be numerous.
   - The follicles are typically atrophic or absent.
   - By IHC, **CD3 is positive** in the paracortical expansion of T cells.
   - The immunoblasts are CD30+, and can be of B or T cell lineage.

RITUXIMAB

- Rituximab is a monoclonal antibody that targets the CD20 receptor on the surface of the B cells.
- It is used in most B cell NHL treatment.
- Rituximab causes cell death by:
  1. Inducing apoptosis,
  2. Antibody-dependent cytotoxicity, and
  3. Complement-mediated cytotoxicity.
- It induces a depletion of B cells.
- The lymph nodes are decreased in size, and the germinal centers become atrophic.
- Most follicles present are primary follicles.
- The sinuses and T cell zones are increased in size.

METHOTREXATE (MTX)

- It is an antimetabolite chemotherapeutic agent.
- MTX can produce reactive hyperplasia to frank lymphomas.
- It is a potent immunosuppressant that activates lytic replication of the EBV in host cells.
- Many lymphoproliferative disorders associated with MTX appear to regress on discontinuation of medication
- Regression usually occurs after **4 weeks** of therapy discontinuation.
- Most large studies have concluded that there is no increased risk of lymphoma in patients undergoing therapy with MTX.
- **Histological findings:** Histological findings are variable depending on the lymphoma that is associated.
  - ***DLBCL*** shows sheets of immunoblasts and centroblasts.
  - Areas of geographic necrosis can be seen too.
  - IHC shows expression of the B cell markers CD20, CD79a and PAX-5.
  - ***Classic Hodgkin lymphoma (CHL)*** shows the presence of Reed-Sternberg cells and other features of CHL.
  - The tumor cells are positive for CD30, CD15, and weakly PAX-5+.
  - CD45 is negative and EBV is frequently positive.
  - ***Hodgkin-like LPDs*** resemble, but do not fulfil the criteria for CHL (CD20+, CD45+).

- The polymorphic/lymphoplasmacytic forms of LPD show partial effacement of the architecture due to a polymorphous infiltrate in the interfollicular areas.
- Small lymphocytes, immunoblasts, small plasma cells, histiocytes and Hodgkin-like cells are present.
- EBV positive cells are present.

SULFASALAZINE

- Lymph node changes can be seen following administration of this drug.
- Hypersensitivity syndrome associated with this drug is also referred to as '3-week sulfasalazine syndrome'.
- Patients typically develop fever, arthralgias, rash, polyarteritis, nephrotoxicity, lymphadenopathy, eosinophilia, lymphocytosis and agranulocytosis.
- The affected lymph nodes show the changes of DIHS.
- **Lymph node morphology:**
  - Disruption of the architecture with LN enlargement.
  - Necrosis, increased number of immunoblasts, and vascular proliferation are also noted.
  - Small lymphocytes and abundant plasma cells are present.
  - An increased number of eosinophils and erythrophagocytosis may also be identified because of the increased vascularity.
  - The term 'angioimmunoblastic lymphadenopathy' was used in the past.

CLOFAZIMINE

- Clofazimine is a medication used in the treatment of leprosy, and sometimes in cases of lupus erythematosus.
- Deposition of crystals of the drug clofazimine in the histiocytes of LN leads to a distinctive-appearing lymphadenopathy.
- It has a high tissue distribution throughout the body and **crystalline** forms of the medication may be deposited in various anatomic sites, particularly the **reticuloendothelial organs**.
- **Lymph node microscopy:**
  - Markedly increased number of histiocytes.
  - Reddish-orange crystal deposits within the cells.

ANTI-TNF-α MEDICATIONS

- These are associated with lymphoproliferative disorders, infections and vasculitis.
- The drugs include infliximab, etanercept, abatacept, and adalimumab.
- They are used to treat autoimmune conditions like psoriasis, Crohn's disease, UC, and RA.
- The first association was seen in **hepatosplenic T cell lymphomas** which is an extremely rare tumor.
- Subsequently, DLBCL, classical Hodgkin's lymphoma and marginal zone lymphoma were reported.
- One particular common finding is the expression of EBV in the tumour cells in cases of DLBCL and classical Hodgkin's lymphoma.

POINTS TO REMEMBER

- Autoimmune disorders could affect the lymph nodes and potentially mimic B and T cell lymphomas.
- Some of these disorders are more characteristic of individuals in the pediatric age group (ALPS, Kawasaki disease), while others are present in older individuals (RA, SLE, sarcoidosis).
- A common finding that groups all of these disorders together is the overall relative preservation of the architecture, a feature that can be particularly helpful in distinguishing them from many B and T cell lymphomas.
- Certain medications may also be responsible for lymphadenopathies.
- Differentiation from lymphoma is very important in these cases.
- Discontinuation of the offending drug often leads to resolution.

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20 Recent Advances in Barrett Esophagus

Harsh Batra

NORMAL ANATOMY

- Esophagus is a fibromuscular tube measuring 18–25 cm in length.
- It starts at the level of C6 vertebra (behind the cricoid cartilage) and enters the diaphragm at T10 vertebrae level.
- It ends at the cardia of the stomach (T11 vertebral level).
- The gastro-esophageal junction lies at the lower end of the esophagus.
- The pink color of the esophageal mucosa contrasts to the deeper red of the gastric mucosa.
- The mucosal transition is marked by an irregular zigzag line called the z-line.
- Histological examination of the gastresophageal (GE) junction shows abrupt transition between the nonkeratinized stratified squamous epithelium of the esophagus and the simple columnar epithelium of the stomach.
- Normally, the cardia of the stomach is immediately distal to the z-line.
- When the anatomy of the mucosa is distorted (e.g. Barrett's esophagus), the true GE junction can be identified by the upper limit of the gastric folds rather than the mucosal transition.

Normal Histology

- Esophagus shows the following layers:
  - *Mucosa:*
    - It is composed of non-keratinizing stratified squamous epithelium.
    - Lamina propria has esophageal cardiac glands similar to cardiac glands of stomach.
    - They are most frequent in the terminal part of the organ.
    - Muscularis mucosa is noticeably thicker than anywhere else in GIT.
    - It is made up of only longitudinal smooth muscle fibers.
  - *Submucosa:* It has mucus secreting esophageal glands.
  - *Muscularis propria:* It shows two types of muscles:
    - Striated (in upper third) and
    - Smooth muscle (in lower third).
    - The middle third contains a mixture of the two muscle types.
  - The esophagus also has an *adventitia*, but not a serosa—distinct from many other structures in the gastrointestinal system

Barrett's Esophagus (BE)

- **Definition:** It is a condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus.
- It is a precursor lesion of esophageal adenocarcinoma.
- There are three types of columnar epithelium:
  - Gastric-fundic type
  - Cardia-type
  - Intestinal-type including goblet cells.
  - However, only the last type has been clearly linked to an increased risk of malignant progression with an annual risk of esophageal adenocarcinoma of 0.5% per year in patients with intestinal metaplasia of the esophagus
- American Gastroenterology Association (AGA) and the American College of Gastroenterology (ACG) currently recommend that:
  - Although columnar-type mucosa can be recognized during endoscopy.
  - **Presence of intestinal metaplasia must be confirmed by biopsy before rendering a diagnosis of Barrett's esophagus.**
- Definitive diagnosis on the grounds of histopathology requires the presence of glandular metaplasia associated with native esophageal structures such as esophageal gland ducts or submucosal glands.
- This indicates that glandular metaplasia is in the esophagus.

Risk Factors

- Male sex
- Smoking
- Middle age
- Caucasian
- Overweight
- Alcohol
- Family history.

- The cell of origin remains unknown, however, theories regarding the cell of origin include:
  - Multipotential stem cells at the basal aspect of squamous esophageal mucosa
  - Cells lining the esophageal gland ducts.
  - Upward migration of gastric mucosa.
- **Screening:**
  - Presence of GERD alone is not sufficient to recommend screening.
  - Up to 40% of patients who have adenocarcinoma of the esophagus have no history of chronic GERD.
  - Difficulties recognizing mucosal lesions, sampling error and disagreement over pathologic interpretation pose a challenge in effective screening.
  - The decision to screen should be individualized for a case.
- Endoscopy shows asymmetric, irregular, salmon-pink tongue-like mucosal projections extending up into the stratified squamous epithelium above the oesophagogastric junction (Fig. 20.1).
- Four quadrant biopsies are taken along every 2 cm of the BE-type mucosa (Seattle protocol).

Fig. 20.1: Barrett's esophagus showing tongue-like mucosal projections

- While this approach samples only a small fraction of the affected lining, it allows the opportunity to **recognize dysplasia and focus subsequent biopsies on the appropriate area** if dysplasia is identified.
- Pathology report of biopsies of the esophagus should always state whether goblet cells (Fig. 20.2) are present in tissue samples obtained from the above GEJ.
- This is because they are present in the mucus secreting glands of the intestine and are *not* present in the mucus secreting glands of the stomach.

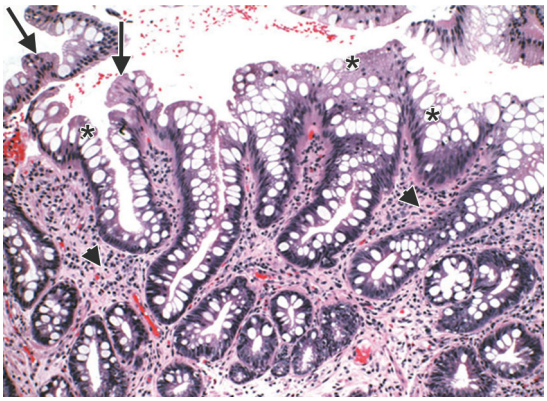

Fig. 20.2: GE-junction biopsy showing goblet cells (asterisk), columnar cells (arrow) and lymphoplasmacytic infiltrate (arrow-heads)

- Based on length of the islands of columnar epithelium Barrett's esophagus has been classified as follows:
  - Long segment (if the tongues are 3 cm or more in length)
  - Short segment (less than 3 cm) and
  - Ultra-short segment (less than 1 cm).
- The exact location of the biopsy relative to the Z-line and oesophagogastric junction is important to know as ultra-short BE can be difficult to differentiate from an irregular oesophagogastric junction.
- It carries significantly lesser risk of cancer development than traditional BE.

Intestinal Metaplasia (IM)

- IM is histopathologically divided into two subgroups based on the mucin content of the columnar-type and goblet cells.
- **Type 1:** Complete—nearly identical to that of small bowel, villi and crypts. Sialomucin predominant.
- **Type 2:** Incomplete
  - Type IIA: Columnar cells secreting neutral mucins or traces of sialomucins.
  - Type IIB: Columnar cells secreting sulphomucins. It has the highest incidence, fairly extensive and has association with other metaplasia.
- IM types IIA and IIB are commonly referred to as incomplete IM or specialized columnar epithelium (SCE).
- They are easily recognised with simple histochemical stains such as alcian blue (pH 2.5)-periodic acid Schiff.
- SCE is the histological hallmark of Barrett's esophagus.
- The intestinal metaplasia is '**specialized**' as it is **unique to the columnar lined oesophagus (CLO)** and it differs from that seen in the stomach.
- **MUC 1** and **6** have been **90% specific** for goblet cells in CLO compared to that of stomach.
- Areas of inflammation in the lamina propria show regenerative changes, viz. mucin depletion, hyperchromatic nuclei, cell stratification and increased mitotic figures which can resemble low grade dysplasia.

The Prague CM Criteria

- The length-based classification of BE (long, short and ultrashort segment) was a bit crude in terms of inter-observer reproduction.
- Therefore, the Prague classification system that measures the circumferential (C) and maximal (M) extents for endoscopic standardization of BE lengths was developed.
- It was finally introduced by the International Working Group for the Classification of Oesophagitis (IWGCO) in 2004.
- Recently, Kinjo et al. suggested that recognition of ultra-short segment BE may be improved using the Japanese EGJ reference point—**the distal end of the palisade-shaped longitudinal vessels**—rather than the traditional proximal limit of the linear gastric mucosal folds currently utilized in the Prague CM criteria.

Histopathology

- Not only a characteristic endoscopic appearance but also histologically confirmed intestinal metaplasia consisting of columnar epithelium with well-formed goblet cells are needed for a diagnosis.
- The spectrum of changes seen has been divided into four clinically significant groups:
  - Negative for dysplasia
  - Indefinite for dysplasia
  - Low-grade dysplasia
  - High-grade dysplasia.
- Despite concerns over adequate sampling and imperfect intra- and interobserver reproducibility (particularly at the low end of the dysplasia spectrum), histologic evaluation for dysplasia retains a key role in the surveillance of patients with BE.

- Patients with histologically confirmed dysplasia are at significantly increased risk of progression to esophageal adenocarcinoma.
- The degree of dysplasia is determined by:
  - Cytology (nuclear and cytoplasmic features).
  - Architecture (relationship of glands and lamina propria).
  - Degree of surface maturation (comparison of nuclear size within crypts to nuclear size at the mucosal surface).
  - Interpreting these findings in conjunction with the amount of background inflammation.

Histological Categories

I. Negative for dysplasia

	Histology	*Follow-up*
Architecture	⅄ Normal with well-spaced glands	⅄ Repeat EGD within 1 year ⅄ EGD every 3 years
Cytology	⅄ Regular nuclei ⅄ Smooth membranes	
Maturation	⅄ Complete	

II. Indefinite for dysplasia

	Histology	*Follow-up*
Architecture	⅄ Normal to mild distortion, often inflamed	⅄ Repeat EGD within 6 months ⅄ Follow-up as indicated by results ⅄ PPI prior to repeat biopsy if significant inflammation present
Cytology	⅄ Hyperchromasia ⅄ Overlapping nuclei ⅄ Irregular nuclear borders	
Maturation	⅄ Complete when intact surface epithelium is present	

III. Low grade dysplasia

	Histology	*Follow-up*
Architecture	⅄ Normal to mild distortion ⅄ Gland crowding	⅄ Repeat EGD within 6 months to rule out high grade ⅄ Expert pathologist confirmation ⅄ Yearly EGD until no dysplasia
Cytology	⅄ Minimal pleomorphism ⅄ Maintained polarity ⅄ Increased mitotic activity	
Maturation	⅄ Minimal to none	

IV. High grade dysplasia

	Histology	*Follow up*
Architecture	⅄ Mild to marked distortion ⅄ Crowded glands ⅄ Cribriform or budding glands	⅄ Repeat EGD within 3 months to rule out cancer ⅄ Expert pathologist confirmation ⅄ Mucosal irregularity–EMR ⅄ Individualized surveillance or treatment plan
Cytology	⅄ Loss of polarity ⅄ Markedly enlarged nuclei ⅄ Prominent pleomorphism ⅄ Atypical mitoses	
Maturation	⅄ None	

- Although difficult, a co-existing esophageal adenocarcinoma should always be ruled in case of high grade dysplasia.
- Suggestive architectural changes include:
  - Single cells in the lamina propria
  - Desmoplasia
  - Cribriform or solid tubular architecture
  - Dilated tubules filled with necrotic debris
  - Extensive neutrophilic infiltrate within the epithelium.
  - Ulcerated high-grade dysplasia
  - Neoplastic tubules incorporated into the overlying squamous epithelium
- A difference between reactive change and true dysplasia sometimes poses a challenge.
- Abrupt transition between normal and abnormal epithelium favors true dysplasia.
- Epithelial changes due to inflammation are more prominent in inflamed foci.
- For all other cases, "indefinite for dysplasia" term can be used.

Surveillance of Barrett's Esophagus

- Ideally, performed after reflux symptoms are controlled, thereby reducing inflammation.
- **Four quadrant biopsies** should be obtained at a minimum of **every 2 cm.**
- There is a significant improvement in survival for cancers that are detected endoscopically versus those that present with symptoms.
- Esophageal adenocarcinoma (EAC) has been shown to have a poor 5-year survival.
- The surveillance intervals suggested by the 2008 ACG Guidelines are dependent on the pathology results.
- Initial biopsy diagnostic of BE:
  1. *Negative for dysplasia:* Repeat within a year.
     If, the second study is also negative for dysplasia, follow-up at 3-year intervals
  2. *Low-grade dysplasia:* Confirm by second opinion
     - Repeat within 6 months to rule out a higher grade of dysplasia.
     - If no higher grade—yearly follow-up until two consecutive exams are negative for dysplasia.
  3. *Indefinite for dysplasia:* Similar to low-grade dysplasia.

4. *High-grade dysplasia:* Confirm with a second opinion, but repeat within 3 months.
   - Biopsies at smaller, 1 cm intervals.
   - If any mucosal irregularities are seen endoscopic mucosal resection is performed to obtain enough tissue for accurate diagnosis.

Improvements In Diagnosis and Surveillance

1. Enhanced Endoscopy

High-resolution white light endoscopy: It combines the following:

- Endoscopes with large numbers of pixels (600,000 to 1,000,000).
- Magnification devices, and
- High-definition screens.
- This leads to greater sensitivity in the detection of early neoplastic lesions.

2. Chemo Endoscopy

This technique uses the application of chemicals that selectively react with and highlight various mucosal features.

A. **Methylene blue**
- 1% sterile solution of methylene blue which acts as the absorbing stain is used.
- Methylene blue is taken up by actively absorbing tissues like small intestinal and colonic epithelial tissue.
- It does not stain non-absorptive squamous mucosa or gastric mucosa and thus helps in detection.
- However, some studies suggest that it may cause DNA damage in Barrett's esophagus.

B. **Indigo carmine dye:**
- It provides a blue contrast stain to highlight the villiform appearance of intestinal metaplasia.
- It is combined with magnification endoscopy to distinguish mucosal pit patterns.
- While there is a good association of certain patterns with intestinal metaplasia, it has not been shown to increase the detection of dysplasia beyond that of high-resolution endoscopy.

C. **Lugol's solution:**
- It is an iodine-based absorptive stain.
- It has an affinity for glycogen in non-keratinized squamous epithelium giving green-brown color within moments of application.
- Absence of staining signifies diminished or absent glycogen content.
- It can be used to identify:
  - Squamous cell carcinoma
  - Dysplasia
  - Barrett epithelium
  - Gastric metaplasia
  - Some degrees of inflammatory esophagitis.

D. **Toluidine blue:**
- It is a basic absorptive dye.
- It preferentially stains nuclei.
- It stains tissue that has increased mitotic activity.

- Most commonly used for identifying esophageal squamous cell carcinoma.
- Positive staining identifies intestinal and gastric metaplastic tissue equally (Sensitivity: 98% and Specificity: 80%).

Advanced Imaging and Detection

- Standard protocol biopsies have been associated with significant miss rates of dysplastic lesions in patients with BE.
- Thus, a variety of methods to optimize the imaging of BE have been developed to improve the efficiency and diagnostic yield of surveillance endoscopy in detecting early neoplasia.
- These techniques use changes that occur at macroscopic, microscopic, and subcellular levels in early neoplasia.
- Three techniques that are worth mentioning are:
  - Narrow band imaging
  - Autofluorescence imaging
  - Confocal laser endomicroscopy

1. **Narrow band imaging:**
   - It uses optical filters to highlight vascular patterns on the mucosal surface and submucosa
   - Its sensitivity is 97% and specificity is 94%.
   - Overall accuracy is 96% in differentiating dysplasia from non-dysplastic Barrett's mucosa.
   - There is a good correlation of vascular patterns identified by magnified NBI with respect to BE and high grade dysplasia.
   - Studies comparing the actual diagnostic yield of NBI to standard endoscopy have given mixed results.
   - However, it has been shown that fewer biopsies are required with this technique in use.
2. **Autofluorescence imaging:**
   - It utilizes differences in the endogenous fluorophores found in normal and neoplastic epithelia.
   - It has a good sensitivity for high grade dysplasia.
   - It has a poor specificity with false positive rates up to 81%.
3. **Confocal laser endomicroscopy (CLE):**
   - Confocal laser endomicroscopy is based on the principle of tissue illumination with a blue laser light (488 nm wavelength) after application of fluorescence agents which can either be applied topically (e.g. acriflavine hydrochloride, cresyl violet) or systemically.
   - Currently, two different endomicroscopy systems are commercially available:
     - One is integrated into the distal tip of a standard, high-resolution endoscope (iCLE; Pentax, Tokyo, Japan) and
     - Another is probe-based (pCLE; Cellvizio, Mauna Kea Technologies, Paris, France).
   - One can do real-time observation and post-hoc analysis of the tissue at about 1000-fold magnification.
   - Analysis of the crypt architecture and capillaries during endoscopic examination can be done.

- The quality is comparable to standard histopathology.
- Initial studies show accuracy rates of >85% in detecting high-grade dysplasia with good interobserver agreement

Advantages of CLE

- It provides real-time microscopic imaging.
- Avoids/reduces the need for random biopsies.
- Allows the clinician to enable or alter targeted therapy like:
  - Endomucosal resection rather than biopsy
  - Postpone ablation.
- It gives the status of resection margins.

Disadvantages

- Currently there is no definitive evidence that CLE provides additional information beyond careful examination using high-resolution white light endoscopy.
- It requires specialized equipment and training that may not be available outside of specialty centers.

Histochemical and Immunohistochemical Stains

Alcian Blue

- It is a positively charged, very large molecule that stains for acid mucopolysaccharides.
- It binds negatively charged low-density mucin found in the goblet cells and stains them blue in color.
- At pH of 2.5, it stains for both sulfated (sulphomucins) and carboxylated (sialomucins) mucopolysaccharides which are found in the goblet cells located in the intestine.
- With incomplete IM, Alcian blue stain highlights the acid mucin of goblet cells blue.
- However, the alternating areas of neutral gastric foveolar-type mucin appear as magenta-colored ''caps'' within the columnar cells.
- With complete IM, which is more frequently encountered in the stomach, Alcian blue stain highlights goblet cells, which are blue, but a foveolar neutral mucin cap is absent.

Immunohistochemical Markers

- MUC1 and MUC6—90% specific but less sensitive for detection of BE.
- CK7, CK8, CK18—stain glandular epithelium.
- CK20, CK13—stain squamous epithelium.
- Studies have shown that Barrett's mucosa displays CK20 immunoreactivity of the surface epithelium and superficial glands with absent staining in the deep glands.
- However, CK7 strongly highlights both the superficial and deep glands.

Biomarkers

- Categorization of dysplasia suffers from interobserver variability.
- Panel of biomarkers like α-methylacyl-CoA racemase (AMACR), β-catenin, cyclin D1 and p53 have shown usefulness in separating grades of dysplasia and distinguishing neoplastic lesions from reactive changes.

AMACR

- **Alpha-methylacyl-CoA racemase** (AMACR) catalyzes racemization of alpha-methyl, branched carboxylic coenzyme A thioesters.
- It is overexpressed in a variety of neoplasms, such as **prostate** and **colon** cancer.
- In BE, AMACR is negative in non-dysplastic foci.
- It is significantly increased in foci of low-grade dysplasia, high-grade dysplasia and adenocarcinoma
- Its sensitivity to detect low grade dysplasia is 96% and high-grade dysplasia is 80%
- Its specificity is 100%.

TP53

- Wild type is seen in non-dysplastic Barrett's esophagus.
- Overexpression of p53 has been shown to help in progression to adenocarcinoma.

Other Markers

- Aneuploidy, increased tetraploidy and loss of heterozygosity for chromosome 17p.
- Loss of heterozygosity for chromosome 17p has been shown to be a good predictor for neoplastic progression even in low-grade/absent dysplasia.
- These studies have utilized flow cytometry to detect DNA content abnormalities in fresh frozen tissue which is not practical in a routine clinical setting.
- **Fluorescence *in situ* hybridization** can be used to detect abnormalities in fixed tissue and most initial studies show promising results.
- So, biomarkers may prove to be the best predictors of cancer progression.
- None of them have been validated in prospective controlled clinical trials.
- Thus, while being promising, they should not replace grading dysplasia for risk stratification in routine clinical practice at present.

Management

Treatment Options

- Anti-reflux therapy
- Surveillance biopsies
- Endoscopic mucosal resection
- Endoscopic ablation
- Usually no surgery

Endoscopic Mucosal Resection

- It is the most widely used treatment technique for Barrett's esophagus.
- The target lesion is first lifted by a fluid (saline or diluted epinephrine) into submucosal layer.
- Subsequently, a transparent cap is attached to the endoscope to suck out the lesion.
- This results in a pseudopolyp that can be captured by a snare.
- The lesion is then removed by electrocoagulation.

Ablation

- It involves the destruction and removal of mucosa.
- Mechanisms include heating, cooling and excitation of tissue to vaporization/coagulation.
- Endpoint is irreversible cell injury and death.
- **Radiofrequency ablation (RFA):** A cylindrical balloon is inflated to bring electrodes in contact with the esophageal mucosa.
- Involves only surface epithelium.
- Appropriate for ablation of Barrett's without dysplasia.
- It is not appropriate if there is evidence of cancer.

Cryotherapy

- Rapid freezing causes failure of cellular metabolism due to stress on lipids and proteins.
- Continued freezing produces extracellular ice, creating a hyperosmotic extracellular environment and drawing fluid from the cell.
- There are two available techniques:
  - *Liquid nitrogen:* Requires large diameter venting tube at minus 196°F temperature.
  - *Carbon dioxide gas:* A much lower profile venting system is needed at minus 78°F temperature.
- Depth of injury by cryoablation depends upon:
  - Duration of spray in seconds
  - Number of repeat cycles.
- There are two phases to depth of injury:
  - *Immediate effect:* Characterized by mild submucosal haemorrhage.
  - *Delayed effect:* Seen as an inflammatory response.

POINTS TO REMEMBER

- Barrett's esophagus (BE) is an increasingly prevalent condition.
- It can lead in some patients to the development of esophageal adenocarcinoma (EAC).
- Various strategies are available to detect BE and further risk-stratify individuals to their cancer risk.
- However, lack of our understanding of the pathogenesis limits our ability to accurately predict who harbors this cancer precursor and who will progress to EAC.
- Special stains and immunohistochemistry may be of some help in this regard.
- There are medical, endoscopic, and surgical treatments that may reduce cancer risk.
- However, appropriate use of these interventions will require additional data about their long-term effectiveness, safety, and costs.

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21 Molecular Pathways in Gliomagenesis

INTRODUCTION

- A **glioma** is a tumor that arises from the glial cells.
- **Glial** in Greek means glue.
- These cells are supporting cells in the central nervous system.
- They surround neurons and provide support and protection.
- The various glial cells are:
  - Astrocytes
  - Oligodendrocytes
  - Ependymal cells
  - Microglial cells.
- The incidence of gliomas is lower in India as compared to the Western world.
- However, it is increasing every year.
- The basic treatment includes surgery plus radiation therapy.
- No single therapeutic agent has been more effective than surgery and radiation therapy.
- However, neurologists, neurosurgeons and neurobiologists think that effective treatments can be developed once the genetic and molecular pathways are better understood.

Molecular Pathways involved in Gliomas

These include the following:

- Abnormalities of cell cycle machinery:
  - p16-cdk4-pRb cell cycle pathway
  - ARF-MDM2-p53 cell cycle pathway.
- Abnormalities of growth factor and growth factor receptor signaling:
  - EGFR
  - PDGFR
  - FGFR.
- Role of PTEN and NF-1.
- IDH1/2 mutations.
- Molecular pathways in ependymomas.

Abnormalities of Cell Cycle Machinery

- These abnormalities usually involve chromosome 9p 21.
- This genetic locus on chromosome 9p encodes two proteins: p16 and ARF.
- These regulate p16-cdk4-pRb and ARF-MDM2-p53 cell cycle pathways respectively.
- These pathways show abnormalities in gliomagenesis.
- Inhibition of p16 leads to an increased level of hypophosphorylated Rb which further causes increase in cyclin A levels.
- On the other hand, inhibition of ARF-MDM2 leads to increased levels of cyclin B and CDK1.
- Both of these eventually cause the promotion of cell cycle.
- p53 (17p) loss of heterozygosity is most commonly seen in grade II –IV astrocytomas.
- Animal studies conducted on mice have shown that abnormalities in ARF function caused a high incidence of glioma formation.
- MDM2 (murine double mutant 2) amplification and overexpression is seen in 8–10% of high-grade astrocytomas.

Abnormalities of Growth Factor and Growth Factor Receptor Signaling

EGFR (7p12)

- EGFR mutation is a major contributing factor in gliomagenesis.
- These abnormalities will lead to EGFR molecules getting constitutionally active without the presence of activating EGF ligand.
- Amplification of EGFR is seen in ~40% cases of glioblastoma multiforme.
- Studies have also shown that overexpression of EGFR in glioma cells inhibited apoptosis due to the upregulation of pro-survival protein Bcl-X.

PDGFR

- Amplifications and overexpression of PDGFR have been observed in glioblastoma multiforme.
- These abnormalities will lead to PDGFR molecules getting constitutionally active without the presence of activating PDGF ligand.
- This leads to increased cell proliferation and prolonged survival.

FGFR

- The mechanisms are the same as described above for EGFR and PDGFR.

Role of PTEN and NF-1

PTEN (Phosphotase and Tensin Homologue)

- It is a tumor suppressor gene.
- Location is on chromosome 10q23.
- Deletion of a part or all of chromosome 10 is one of the most frequent genetic abnormalities in gliomas.
- Studies have shown increasing incidence of chromosome 10 loss with increasing grades of astrocytomas:
  - Astrocytomas—35% loss
  - Anaplastic astrocytomas—66%
  - Glioblastoma multiforme—93% loss.

- Loss of heterozygosity is seen in ~ 74% cases.
- Other mutations such as deletions are seen in 44% cases.
- Studies have shown that high levels of PTEN correlated with better prognosis of patients.

NF-1 (Neurofibromin)

- It is located on chromosome 17q11.2.
- Loss of heterozygosity is the most common molecular abnormality.

Isocitrate Dehydrogenase Mutations

- Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate, producing α-ketoglutarate and CO_2.
- It has 3 isoforms: IDH1, IDH2 and IDH3.
- IDH3 catalyzes the third step of the citric acid cycle within the mitochondria.
- IDH1 and IDH2 catalyze this same reaction, but outside of the context of the citric acid cycle.
- IDH3 reduces NAD+ to NADH in this process, whereas IDH1 and IDH2 use NADP+ instead.
- IDH1 is the only isoform localized to the cytoplasm.
- When mutations occur in IDH1 or IDH2, the enzyme develops a preferential affinity for α-ketoglutarate instead of isocitrate.
- This leads to the production and accumulation of the oncometabolite 2-hydroxy-glutarate (2HG).
- Both IDH1/IDH2 mutations are missense mutations.
- IDH1 R132 mutation: At position number 132 arginine is replaced by histidine.
- IDH2 R172 mutation: At position number 172 arginine is replaced by histidine.
- The exact pathway to oncogenic potential still remains controversial.
- However, the current favored hypothesis is shown in Fig. 21.1.

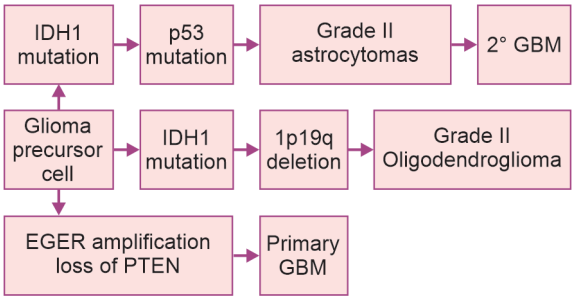

Fig. 21.1: IDH mutations in gliomagenesis

- As shown in Fig. 21.1, IDH1 mutation in glioma precursor cell followed by p53 mutation leads to grade II astrocytomas followed by secondary glioblastoma multiforme (GBM).
- IDH1 mutation followed by 1p19q deletion leads to Grade II oligodendroglioma.
- However, EGFR amplification and loss of PTEN cause primary GBM.
- The above facts have great clinical relevance.

- IDH mutated gliomas have significantly better prognosis and survival as compared to IDH wild type.
- Based on these findings the immunohistochemical approach to gliomas is significantly changed.
- The current approach to low-grade gliomas is shown in Figs 21.2A and B.

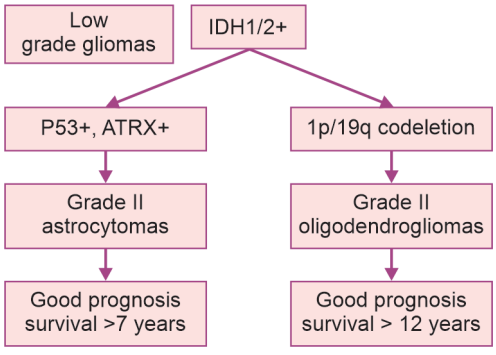

Fig. 21.2A: Immunohistochemical approach to low-grade glioma (IDH1/2+)

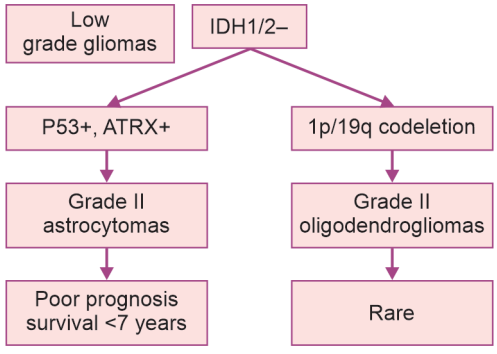

Fig. 21.2B: Immunohistochemical approach to low-grade glioma (IDH1/2-)

- The approach to grade III astrocytomas is shown in Figs 21.3A and B.

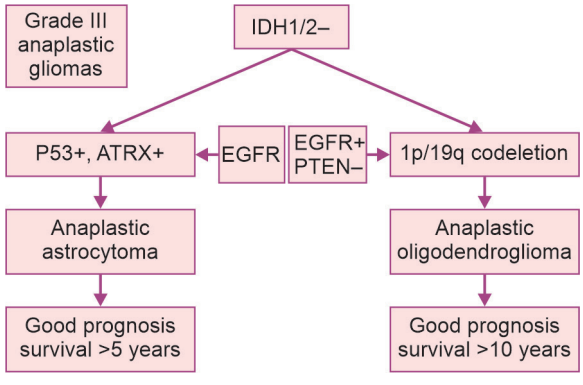

Fig. 21.3A: Immunohistochemical approach to grade III astrocytoma (IDH1/2+)

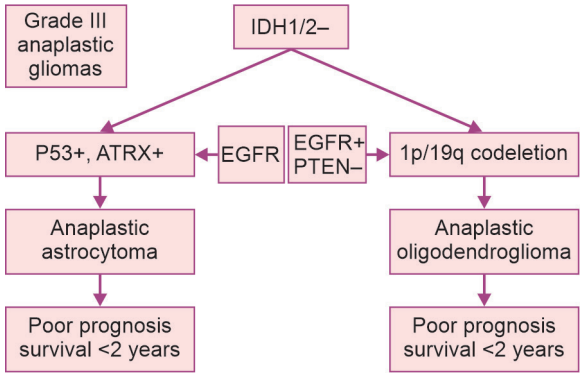

Fig. 21.3B: Immunohistochemical approach to grade III astrocytoma (IDH1/2–)

- The diagnostic approach to GBM is shown in Fig. 21.4.

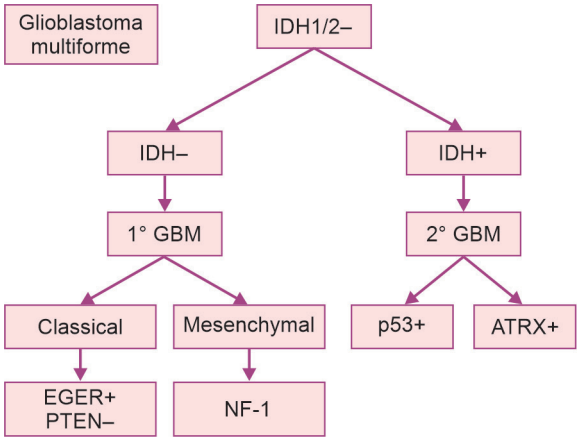

Fig. 21.4: Immunohistochemical approach to GBM

- Also, in light of the greater understanding of molecular pathways of gliomagenesis, the diagnosis of oligoastrocytoma is strongly discouraged by WHO.
- In all cases morphologically looking like oligoastrocytoma, it is recommended to do the following:
  - IDH1/2
  - ATRX
  - TP53
  - 1p/19q codeletion
- The results are interpreted as follows:
  - IDH+, ATRX-, TP53+ : IDH mutant astrocytoma
  - IDH+, ATRX+, TP53+ : IDH mutant astrocytoma
  - IDH+, ATRX-, TP53+, 1p/19q codeletion+ : Oligodendroglioma
  - IDH- : IDH wild type astrocytoma

Ependymoma

- The major molecular pathways implicated in ependymoma are as follows:
  - MAP kinase (MAPK)
  - EGFR
  - PDGFR
  - NOTCH: Tumor progression
- Out of these, NOTCH pathway is most important as it promotes tumor progression.
- NOTCH functions as a receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell fate determination.
- Upon ligand activation through the released notch intracellular domain (NICD), it forms a transcriptional activator complex with RBPJ/RBPSUH and activates genes of the enhancer of split locus.

Gliomas and Micro-RNA

- The most important micro-RNAs that play a role in gliomagenesis are miR-26a and miR-34a.

- miR-26a causes downregulation of PTEN, RB and MAPK pathways.
- miR-34a downregulates CDK6, MET, NOTCH1 and NOTCH2.
- These actions lead to gliomagenesis.
- Other micro-RNAs which have a possible role in glioma formation are miR-7, miR-21, miR-124a, miR-137 and miR-222.

POINTS TO REMEMBER

- In recent years the understanding of molecular pathways of gliomas has vastly improved.
- Several novel pathways have emerged as key components in signaling mechanisms that drive the development of gliomas from glial cells or their precursors.
- Active clinical research is presently underway for developing more effective treatments targeting these molecular pathways.
- Pathologists have an extremely crucial role as they have to give accurate diagnosis on the basis of current WHO classification.
- This makes a difference by predicting the prognosis of these patients.
- The promise of novel targeted therapies is expected to revolutionize management of these cases.
- However, still a lot has to be done before this becomes a reality.

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22 Neuroendocrine Tumors of Pancreas—Recent Advances

Jyoti Singh

INTRODUCTION

- The pancreas is divided functionally into:
  - **Exocrine pancreas**
  - **Endocrine pancreas**
- 85% of pancreatic mass is endocrine pancreas**.**
- 2% of pancreatic mass is exocrine pancreas.
- It secretes a clear, alkaline (pH 7–8.3) solution of 1–2 L/day, containing digestive enzymes.
- Secretion is stimulated by the following:
  - Secretin
  - Cholecystokinin (CCK)
  - Parasympathetic vagal discharge.

Endocrine Pancreas

- One million islets of Langerhans in the normal adult pancreas.
- Vary greatly in size.
- Larger islets are located closer to the major arterioles.
- Smaller islets are embedded more deeply in the pancreatic parenchyma.
- Islet cells originate from neural crest cells.

Neuroendocrine Tumor (NET)

- Include a heterogeneous group of neoplasms.
- Arise from cells which produce and secrete hormones.
- Slow growing and malignant, with metastatic potential.
- Characterized by ability to produce peptides that may lead to associated syndromes (functional vs nonfunctional).
- Incidence -1/100000.
- Classified according to WHO (WHO 2017).

Overall GI NET Distribution

- The pancreas is the most common primary location of NET.

CLASSIFICATION OF NEUROENDOCRINE TUMORS

Embryological Classification

- ***Foregut***: Respiratory tract, stomach, duodenum, pancreas.
- ***Midgut***: Bowel up to ascending colon and appendix.
- ***Hindgut***: Remaining colon and rectum.

Histological Classification

- Nodular solid nests.
- Trabecular structures.
- Tubular, acinar or rosette.
- Atypical differentiation.

Biochemical Classification

- Ghrelin
- 5HT (enterochromaffin cells)
- Somatostatin (D)
- Glucagon
- Insulin
- Gastrin
- Secretin
- GIP/Xenin
- Motilin
- Neurotensin
- Histamine

WHO 2017 Classification

- Histopathologic criteria, including ki-67 proliferative index and mitotic index to predict the tumor's grade and biological behavior.
- Definition: Overarching term to encompass all of the pancreatic entities with significant neuroendocrine differentiation (tumors, carcinomas, mixed carcinomas).
- A comparison between WHO 2010 and WHO 2017 classifications is shown in Table 22.1.

Table 22.1: Comparison of the 2017 and 2010 WHO Classification and Grading of the Pancreatic Neuroendocrine Neoplasms

Classification/Grade	*Ki-67 Proliferation Index (%)*	*Mitotic Index*
2017 WHO Tumor Classification		
Well-differentiated panNEN:		
PanNET, G1	<3	<2
PanNET, G2	3–20	2–20
PanNET, G3	>20	>20
Poorly differentiated panNEN: panNEC		
PanNEC, G3 (small cell and large cell type)	>20	>20
2010 WHO Tumor Classification		
Well-differentiated endocrine tumor, GI	<3	<2
Well-differentiated endocrine tumor, G2	3–20	2–20
Poorly differentiated neoplasm: Neuroendocrine carcinoma, G3 (small cell and large cell type)	>20	>20

- G3 NETs with Ki67 20–55% may be well-differentiated biologically.
- Well-differentiated panNET with an elevated proliferative rate has a better prognosis.
- There is no change in TNM classification.

WELL-DIFFERENTIATED PAN NET (G1, G2, AND G3)

- Slow-growing tumors
- Equal sex preference.
- Broad age range, highest incidence peak between third and sixth decade.
- Clinically, these neoplasms present with variable symptoms related to their different clinical secretory syndromes, genetic syndromes, or obstructive behaviors.
- >90% show organoid architecture, well-differentiated cytology with the absence of non-neuroendocrine carcinoma components.
- Most functioning and non-functioning panNETs are graded as well-differentiated.

Functioning PanNET

Functioning panNETs abnormally secrete large amounts of peptide hormones and other bioactive compounds leading to hypersecretion clinical syndromes.

- Gastrinoma
- Insulinoma
- Glucagonoma
- VIPoma
- Somatostatinoma
- PPoma.

Non-functioning PanNET

- Non-functioning panNET is reserved for neuroendocrine neoplasms occurring in patients with no paraneoplastic symptoms related to tumor hormone hypersecretion.
- They may or may not secrete peptide hormones but at levels insufficient to cause clinical symptoms.
- Recent advances in imaging modalities are responsible for an important increased number in the diagnosis of incidental non-functioning panNET.
- This has led, to its relative frequency, in some studies, to reach as high as **70% to 90%.**
- Their symptomatology is usually related to its mass related burden of the primary disease or metastasis.
- Tumors in head of pancreas cause biliary obstruction, gastric outlet obstruction, pancreatitis, postobstructive jaundice, and nonspecific abdominal pain.
- Although not associated with hypersecretory syndromes, non-functioning tumors may express various peptide hormones in immunohistochemical studies, such as glucagon, somatostatin, and serotonin.
- Mostly occur sporadically.
- Nearly 10% are associated with predisposing genetic syndromes, including MEN1, von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1), and tuberous sclerosis complex (TSC).

- Heterogenous group of diseases and range from slow-growing non-infiltrative tumors to rapidly metastasizing malignancies.
- Diagnosed at more advanced stages.
- Present with distant metastases in up to 50% of patients.
- Metastatic disease primarily occurs in the liver, although other sites including bone, peritoneum, adrenal, brain, and spleen have been documented.

MOLECULAR GENETICS OF WELL-DIFFERENTIATED panNETs

MEN1

- Inherited as part of MEN1 syndrome.
- >35% of cases.
- It is a somatic disorder.
- MEN1 gene encodes a protein, menin, a histone methyltransferase complex, which acts as a tumor suppressor.
- Recent advances have shown that the tumor-suppressing pathway mediated by MEN1 is dependent on the Pi3K-Akt-mTOR pathway.
- This is mediated by PHLDA3, a novel tumor suppressor gene, that inhibits Akt activation.

DAXX/ATRX

- Both DAXX (death-domain-associated protein) and ATRX (α thalassemia/mental retardation syndrome X-linked) genes encode proteins implicated in chromatin remodeling.
- More than 45% of pan NET have been found to have a mutation in one of the 2 genes.
- Mutation in these encoding proteins results in alternative lengthening of the telomeres (ALT) phenotype and chromosomal instability.
- Recent advances, have shown that panNETs with somatic inactivating mutation in DAXX/ATRX gene acts as a potential independent prognostic factor.
- It is associated with a poor prognosis.

Poorly Differentiated NET (G3 NEC, Small and Large NEC)

- ~2% to 3% cases.
- Slight male predominance with an incidence peak in the sixth to seventh decade and frequently involves the head of pancreas.
- Their clinical presentation is very similar to exocrine pancreatic tumors and is related to their location, large size and both obstructive and somewhat invasive nature.
- Small cell carcinoma and large cell NE carcinoma show less organoid architecture and absence of G1 or G2 NE components.
- They may have non-neuroendocrine carcinoma components, less diffuse immuno-expression of general NE markers.

Molecular Genetics of panNECs

- Most commonly involved genes include KRAS, PIKC A/PTEN and BRAF.

- Other newer and less frequently found markers include RB1, CDKN2A/p16, APC, FBXW7, WNT, BCL2, and CTNNB1.
- Molecular abnormalities encountered in panNETs (MEN1, DAXX, and ATRX) and ductal pancreatic adenocarcinoma (SMAD4/DPC4) are not seen.
- Abnormal immunostaining pattern for p53 and the Rb indicates that inactivation of these 2 pathways is a central feature for panNEC development.

Pancreatic Neoplasms with Mixed Differentiation

- Neuroendocrine (usually poorly differentiated) plus a nonneuroendocrine component:
  - Mixed ductal neuroendocrine carcinoma.
  - Mixed acinar neuroendocrine carcinoma.
- Each component should be >30%.
- Previous terms:
  - Mixed exocrine–endocrine carcinoma, MEEC (2004).
  - Mixed adenoneuroendocrine carcinoma, MANEC (2010).
  - Mixed neuroendocrine non-neuroendocrine neoplasm, MiNEN.

CLINICAL PRESENTATION

Asymptomatic panNETs

- Tumors without secretory symptoms are not associated with a hormonal syndrome
- They are often found incidentally or due to mass effect.
- Patients present late with large malignancies or advanced disease.
- Symptoms due to tumor growth or spread:
  - Abdominal pain
  - Jaundice
  - Diarrhea
  - Indigestion
  - Weight loss.

Secretory Symptoms Suggesting Specific Functional Tumour

Insulinoma

- >35% to 40% (Table 22.2)
- Arise from insulin and proinsulin producing β cells
- Episodic hyperinsulinemia.
- Whipple's triad:
  - Episodic hypoglycemia,
  - Central nervous system (CNS) dysfunction temporally related to hypoglycemia (confusion, anxiety, stupor, paralysis, convulsions, coma).
  - Reversal of CNS abnormalities by glucose administration.
- >90% are benign and often cured by surgical resection.

Gastrinoma

- 2nd most common functioning panNETs 20% of cases (Table 22.2).

Table 22.2: Pancreatic NETs types, malignant potential and frequency of occurrence			
Tumor	*Islet cell type*	*Hormone produced*	*Malignant potential*
Gastrinoma	gamma	Gastrin	Very high
Insulinoma	beta	Insulin	Low
Glucagnoma	alpha	Glucagon	Very high
VIPoma	delta	VIP	High
Somatostatinoma	delta	Somatostatin	Very high

- Gastrinoma triangle, a region between the junctions of cystic and common bile ducts, second and third parts of the duodenum, and neck and body of the pancreas.
- Gastrin-producing G cells, leading to a clinical syndrome called Zollinger-Ellison syndrome (ZES).
- >90% of gastrinomas are malignant with one-third presenting metastasis at the time of diagnosis.
- Liver and bone are common spread sites.

VIPoma

- 3% to 5% of functioning panNETs.
- Non-β islet cells that produce vasoactive intestinal polypeptide.
- >85% are found in the pancreas, predominantly the tail.
- Hypersecretion syndrome/Verner-Morrison/watery diarrhea, hypokalemia, and achlorhydria (WDHA) are associated with VIPoma.

Glucagonoma

- 5% of panNETs
- Fourth most common functioning panNET
- Glucagon-producing cells
- Glucagonoma syndrome
- Necrolytic migratory erythema (NME), diabetes mellitus, and weight loss
- 3% cases are associated with MEN1 syndrome (Table 22.3)
- More than 70% to 90% are malignant
- They present with metastases at the time of diagnosis, usually involving the liver.

Table 22.3: Syndromes associated with panNETs			
Syndrome	*Inheritance/Gene*	*Chromosome*	*Incidence of pancreatic tumor*
MEN 1	AD/ MEN1	11q13	40–50%
VHL	AD/VHL	3p25	16–17%
NF1	AD/NF1	17q12	<10%
TS	AD/TSC1,TSC2	9p,16p	Rare

Somatostatinoma

- <5% of functioning panNETs.
- Arise from somatostatin-secreting D cells
- Found incidentally.

- The main presenting symptoms are usually related to mass effect.
- Most will commonly present with diabetes mellitus, hypochlorhydria, gallbladder disease (cholelithiasis), diarrhea, steatorrhea, anemia and weight loss.
- Associated with neurofibromatosis type 1 (Table 22.3) and MEN1.
- Recently, HIF2A, a gene involved in the control of cellular response to hypoxia has been implicated.
- Dysregulation of HIF2A leads to polycythemia, paraganglioma, and somatostatinoma syndrome.
- 60% to 70% of these tumors are malignant at the time of diagnosis with two-thirds revealing lymph node or liver metastasis.

Serotonin-producing Tumors

- Extremely rare and ~100 cases have been documented in the literature.
- Arise from either enterochromaffin cells, Kulchitsky cells, or from multipotent precursor cells.
- Serotonin hypersecretion can cause carcinoid syndrome, which has been documented predominantly in advanced metastatic disease.
- Classic triad of cutaneous flushing, diarrhea, and valvular disease.

ACTH-producing Tumors

- Rare and <150 cases have been reported in the literature.
- Ectopic Cushing syndrome, additional clinical features of ZES (34.5%), insulinoma syndrome (5%), and carcinoid syndrome (1 patient), caused by the concomitant release of gastrin, insulin, and serotonin, respectively.
- High level of serum ACTH and β-endorphin may help in the diagnosis.
- Relative frequency: Asymptomatic > Insulinoma > Gastrinoma> Glucagonoma > VIPoma > Somatostatinoma > Others.

DIAGNOSIS

- History and physical exam
- Biochemical markers (serum, tissue, urine)
- Imaging
  - Computed tomography scan (CT)/magnetic resonance imaging (MRI)
  - Nuclear imaging
  - Endoscopic ultrasound (pNET only).

Circulating Tumor Markers

- The frequency of elevated tumor markers is as follows:
  - Chromogranin A: 70–90%
  - Pancreatic polypeptide: 40–55%
  - HCG: 30%
  - NSE: 35%
- Level of chromogranin A correlates with **tumor load**
- It increases over time indicating tumor progression.
- Chromogranin A is falsely elevated in the following conditions:
  - Hepatic failure

  - Proton pump inhibitor use
  - Chronic atrophic gastritis.
- Chromogranin B is superior to chromogranin A.
- Specific markers:
  - Gastrin
  - Glucagon
  - Insulin
- For patients with carcinoid syndrome, urinary 5HIAA is still an important biomarker.

IMAGING

Endoscopic USG

- High sensitivity (76–100%) to localize small and multiple lesions.
- panNETs have high vascularity, therefore, they are better localized.

Contrast Enhanced USG (Ultrasound Microbubble Contrast Medium)

- New technique
- Helps to localize small lesions
- Liver metastasis.

Intraoperative Ultrasound (IOUS)

- Similar advantages like EUS.
- Better sensitivity for identifying small lesions in the head and multiple lesions (97%).
- Useful addition to surgical palpation, particularly in small tumors such as insulinomas.

CT and MRI

- A sensitivity of 94% for pancreatic lesions.
- CT imaging study of choice.
- MRI is more sensitive for detecting small lesions and liver metastasis.

Radionuclide Scan

- Scintigraphy with Octreotide.
- Multiple tumors in patients with familial syndromes.

Newer Imaging Modalities

- PET with 68 Ga DOTATATE
- FDA approved
- PET with FDG radiotracer does not delineate panNETs.

Hybrid Imaging

- CT fusion imaging (The 'Hawkeye' system)
- Combines the advantages of SPECT/PET functional imaging with the anatomical resolution of CT.

Advantages

- Functionally active areas can be co-located with possible sites of disease visible on CT
- Physiological sites of tracer uptake can be clearly identified and thus counted as possible sites of disease.

Morphology

- **Gold standard** for diagnosis.
- The diagnosis of panNET is based on the morphological features.

Gross

Well Differentiated

- Solitary.
- Well-circumscribed neoplasms partially and completely surrounded by a thin capsule.
- Light yellow to reddish tan, occasional cystic changes, and hemorrhage.
- Soft in consistency.

Poorly Differentiated

- Solitary/ Multiple
- Capsule +/–
- Reddish tan and usually present with necrosis, cystic changes, and hemorrhage.

Microscopy

Well Differentiated

- Well-differentiated tumors can manifest a diverse range of histological appearances
  - Nest
  - Trabecular
  - Organoi
  - Tubuloacinar
  - Glandular growth pattern.
- Usually no associated necrosis.
- The tumor cells are round, polygonal to plasmacytoid and spindled.
- The nuclei are relatively uniform and homogeneous, featuring low-to-moderate atypia, with characteristic salt and pepper chromatin, and finely granular cytoplasm.
- The underlying stroma can reveal areas of fibrosis and hyalinization and occasionally, can be richly vascularized.
- In slow-growing tumors, calcifications can be observed.
- In addition, amyloid deposits and psammoma bodies are sometimes encountered in insulinoma and somatostatinoma, respectively.

Poorly Differentiated

- Nested, organoid, or solid growth-like pattern composed of highly atypical small to large polygonal cells with large vesicular nuclei and often prominent nucleoli.
- The small cell type has a more infiltrative growth pattern.

- The large cell type is more commonly observed, accounting for ~60% of cases.
- Areas of geographic necrosis, vascular invasion, and mitoses are easily identifiable.
- Immunohistochemical studies are needed to confirm the diagnosis.

IMMUNOHISTOCHEMISTRY

- The various pan-neuroendocrine markers are shown in Table 22.4.

Table 22.4: Pan-neuroendocrine IHC markers

Pan-neuroendocrine markers	
Cytosolic	NSE, PGP 9.5
Related to secretory granules	Chromogranin
Related to synaptic vesicles	Synaptophysin, VMAT
Intermediate filaments	NF, CK HMW
Adhesion molecules	N-CAM

- NSE and chromogranin are more specific (Table 22.4).

Newer Markers

Neuroendocrine-secretory protein-55 (NESP55)

- A 241 amino-acid polypeptide
- Belongs to the chromogranin family located within the large dense-core secretory granules
- Stains panNETs and distinguishes them from gastrointestinal NETs.

INSM1 (Insulinoma-associated Protein 1)

- INSM1 is the best marker for the diagnosis of neuroendocrine tumors when compared with CGA, SYP and CD56.
- Nuclear positivity.

Treatment

- The three basic principles of therapy are as follows:
  - Surgical therapy
  - Symptomatic therapy
  - Antiproliferative therapy.

Somatostatin Analogs

- Initially developed to palliate hormonal symptoms.
- In recent years, accumulating data have supported the role of SSAs as antiproliferative agents capable of stabilizing tumor growth.
- Drugs:
  - Octreotide
  - Lanreotide.
- Somatostatin (SST) receptors are highly expressed on the surface of GI NETs.
- More than 80% of all NETs express SST receptors.

Targeted Therapies

- Designed to attack some specific aspect of cancer cells
- Targeted therapies:
  - mTOR Inhibitors: Everolimus
  - Tyrosine kinase inhibitors: Sunitinib, sorafenib
  - VEGF inhibitors: Bevacizumab

Newer Chemotherapeutics

- A recently published phase II trial of temozolomide and bevacizumab reported a response rate of 33% in a more heavily pretreated subset of panNETs.

POINTS TO REMEMBER

- **WHO 2017** used the word 'neoplasm' instead of tumor.
- Well and poorly-differentiated neoplasm.
- WHO 2017 introduced the concept of high-grade well-differentiated NEN.
- G3: G3 well-differentiated and G3 poorly-differentiated neoplasm.
- Conversely, this classification system is currently adopted by the AJCC 2017 for all anatomic sites of the digestive system.
- Therapy for these tumors is advancing at a rapid pace.

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Recent Updates in Penile Carcinomas

23

Anam Singh

INTRODUCTION

- Penile carcinoma is more common in the developing countries as compared to the developed Western countries.
- Majority of them are squamous cell carcinoma.
- Penile squamous cell carcinomas are morphologically, etiologically and biologically heterogeneous.
- The overall prognosis is good.
- However, certain subtypes need to be identified as they need more aggressive treatment.

EPIDEMIOLOGY

- Approximately 10–20% cases occur in Asia (excluding Japan). The other areas where it is common are Africa and South America.
- Usually age range is 40–70 years, median age 58 years.
- It is rare if circumcision is done at birth, more common if late circumcision (after age 10).
- More prevalent in populations with lower education and higher poverty.
- More common in Hispanic and black men. Familial cases have occasionally been reported.
- Patients from high-risk areas tend to be younger and present with a higher stage disease.
- Rarely associated with genital piercing.

Risk Factors

- These include the following:
  - Phimosis and long foreskin
  - Paraphimosis
  - Genital warts (6 times increased risk)
  - HPV infection
  - Penile injury, tears and chronic balanitis
  - Smoking, psoriasis patients treated with UV-B radiation
  - Penile rash > 1 month
  - Immunosuppression and radiation therapy.

History

- Paget first described penile epithelioma (1890s).
- Spindle cell carcinoma was reported in 1921 by James Ewing.
- Ackerman described verrucous carcinomas in the oral mucosa in 1948.
- This was followed by a paper on anogenital tumors by Kraus in 1966.
- A major contribution towards anogenital squamous carcinomas, was the identification in 1993 by Kurman of warty and basaloid vulvar carcinomas.
- First comprehensive histological classification of penile carcinomas was published in 1995.
- An attempt to correlate morphological subtypes with HPV was made.
- In addition to previously known subtypes—usual, verrucous, and sarcomatoid carcinomas, warty, basaloid, papillary and mixed carcinomas were noted.
- In the third series Armed Forces Institute of Pathology (AFIP) fascicle, and in the WHO (2003) additional refinements of this schema were done.
- Adenosquamous and clear cell carcinomas were added.
- More recently, the fourth AFIP fascicle edition 2012 included carcinoma cuniculatum, pseudohyperplastic, and pseudoglandular carcinomas.

Recent Updates

- In order to further refine and update the classification of penile carcinoma International Society of Urological Pathology (ISUP) held an expert-driven penile cancer conference in Boston in March 2015.
- It focused on the new WHO classification of penile cancer.
- The conference was preceded by an online survey of the International Society of Urological Pathology members and the results were used to initiate discussions.
- The important points that came up in this conference were as follows:
  - A description of HPV-related neoplasms, which may be recognized by their histological features, was presented.
  - p16 was recommended as a surrogate indicator of HPV.
  - A three-tier grading system was recommended for penile squamous carcinomas; which was also adopted by the WHO (2016).
  - Many of the distinctive histologic subtypes of squamous cell carcinoma are associated with distinct grades, based on the SCC subtype histological features.

WHO CLASSIFICATION 2016

- The major changes in the new WHO classification (2016) of penile carcinoma are as follows:
  - SCC has been divided into non-HPV-associated SCC and HPV-associated SCC.
  - The following new entities have been added:
    - Warty basaloid SCC
    - Papillary basaloid SCC
    - Lymphoepithelioma-like SCC.
  - It is now recommended **not** to use the term **Buschke-Lowenstein tumor** as it erroneously used for large condylomas, verrucous carcinomas, papillary NOS and warty carcinomas.

ROLE OF HPV IN PENILE CARCINOMA

- HPV is a **DNA** virus.
- It has been implicated for its role in cervical, penile anorectal, oropharyngeal and tonsillar cancers.
- HPV encodes—***E5, E6* and *E7*** oncogenes
- E6 and E7 disrupt centrosome synthesis required for mitosis.
- E5 gene product is a transmembrane protein that regulates activation of EGFR.
- EGFR up-regulation causes a decrease in E-cadherin expression and an increase in matrix metalloproteinase **(*MMP*)*-9*** which causes decreased cell-to-cell adhesion.
- E6 inhibits p53 expression.
- E7 displaces Rb from E2F transcription factors which are normally sequestered by Rb.
- High-risk HPV include genotypes: 16, 18, 31, 33, 45, 56, 65.
- Low-risk HPV includes: 6 and 11.
- In case of high-risk HPV infection, viral E7 protein binds to Rb tumor suppressor with much higher affinity than the low-risk HPV subtypes.
- In low-risk HPV, the HPV genome is maintained in a non-integrated episomal form, whereas in high-risk, the genome gets integrated the host genome.

HPV-associated Carcinomas

Basaloid Carcinoma

- It is the most common type of HPV-related penile carcinoma.
- Males in 50s are usually affected.
- This is about 10 years younger than patients with usual squamous cell carcinoma.
- Most common sites involved are glans and prepuce.
- Grossly, there is surface ulceration and a deeply infiltrative tumor mass.
- Cut surface shows characteristic tan solid appearance.
- Microscopy shows small to intermediate basophilic cells with scant cytoplasm, indistinctive cell borders and high mitotic/apoptotic rate (Figs 23.1 and 23.2).

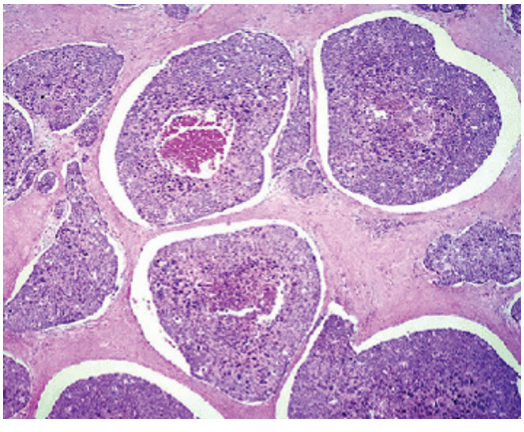

Fig. 23.1: Basaloid carcinoma of penis showing tumor cells in nests give rise to basaloid appearance

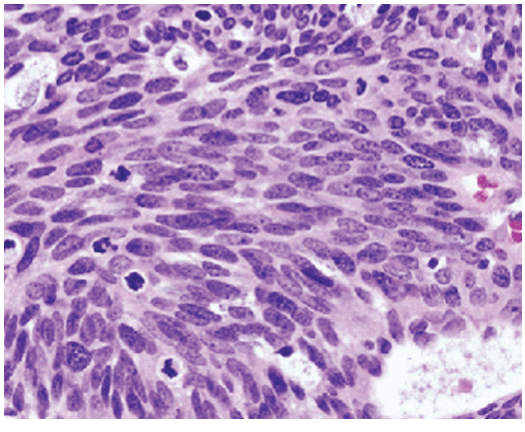

Fig. 23.2: Frequent mitoses and apoptosis

- IHC: p16 positive
- Vascular invasion and deep invasion are frequent.
- About half of patients may present with regional nodal metastasis.
- Mortality varies from 20 to 30%.

Papillary Basaloid Carcinoma

- It is a new variant that has been added to the new WHO classification (2016) of penile carcinoma.
- Rare variant of basaloid carcinomas.
- It is seen in Glans of older males.
- Grossly they are villous and exophytic.
- On cut surface, non-invasive (or surface) component of the lesion is exophytic and papillomatous.
- When invasive, there is often a solid beige tumor mass similar to basaloid carcinomas.
- Microscopic examination shows papillae with a central fibrovascular core with cells that are small and basophilic.
- IHC: p16 positive.

Warty (Condylomatous) Carcinoma

- This is an exophytic verruciform neoplasm.
- Glans, sulcus, or foreskin are the common sites.
- Patients age varies from 40 to 50 years.
- Grossly, cauliflower or cobblestone-like appearance.
- On the cut surface—tan to yellow papillomatous growth with a darker center.
- Microscopy shows, condylomatous papillae with a prominent central fibrovascular core.
- Cells are clear, and parakeratosis and **pleomorphic koilocytosis** are the rule.
- The interface of tumor and stroma is invasive and jagged, rarely non-invasive.
- Inguinal metastases are unusual and mortality is low.

Warty Basaloid Carcinoma

- This is also a new entity added to the recent WHO classification.
- It is a variant of warty carcinoma.
- Mixed tumor with condylomatous and basaloid features.
- Most common site distal penis.
- Age group: 50–60 years.
- Grossly, they are large exo-endophytic tumors.
- The cut surface shows a biphasic papillomatous tumor on the surface and a solid tumor in deeper erectile tissues.
- Microscopy shows areas of warty carcinoma intermingled with others showing basaloid features.
- Areas of basaloid morphology usually predominate.
- Inguinal metastases are not infrequent.
- Mortality is in between that of basaloid and warty carcinoma, although clearly higher than warty carcinomas.

Clear Cell Carcinoma

- It is an aggressive tumor variant of warty carcinoma.
- Most common site: Glans or foreskin.
- Microscopic examination shows a distinctive **nesting pattern, solid or centrally necrotic.**
- Composed of polyhedral to round cells with prominent clear cytoplasm.
- Nodal metastasis may be present.
- Due to its rarity, there is scant information on prognosis and mortality.

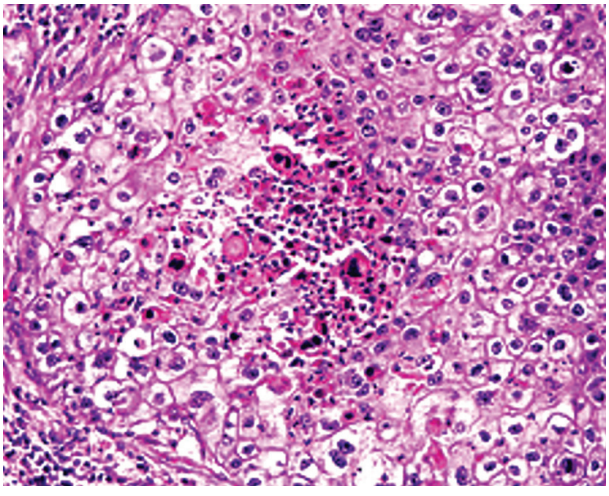

Fig. 23.3: Clear cell carcinoma showing tumor cells in nests with central necrosis. These cells have a prominent clear cytoplasm.

Lymphoepithelioma-like Carcinoma

- It is a new variant that has been added to the updated WHO classification (2016) of penile carcinoma.
- Tumor derives its name from the lymphoepithelioma (undifferentiated nasopharyngeal carcinoma).
- Age group affected is between 50 and 70.
- It presents as a large tumor in glans with an extension to the foreskin.
- Grossly, they are gray white tumor masses mainly affecting the glans.
- Microscopy shows syncytial growth pattern of poorly differentiated to undifferentiated cells.
- Tumor cells are present in the form of irregular geographic sheets/nests/trabeculae/cords.
- A distinctive feature is the presence of an intermixed **dense lymphoplasmacytic and eosinophilic infiltrate, which obscures tumor cell boundaries.**
- A note should be made of the fact that medullary carcinoma of the penis, has also been rarely reported, usually associated with HPV but is not included in the WHO classification 2016.
- The gold standard for HPV detection is PCR, but p16 immunostaining is a useful surrogate.

NON-HPV-ASSOCIATED CARCINOMAS

Pseudo-hyperplastic Carcinoma

- It is a non-verruciform, highly differentiated invasive tumor resembling pseudoepitheliomatous hyperplasia.
- Affects elderly patients.
- The most common site is inner mucosa of the foreskin.
- It is associated with a good prognosis.
- Usually no recurrence after excision is seen.
- It does not metastasize to inguinal nodes.
- Microscopic examination shows downward proliferation of keratinizing nests of squamous cells with minimal atypia.
- Squamous pearls are seen in almost all cases.
- Tumor cells have eosinophilic cytoplasm with distinct intercellular bridges.
- Definite invasion with the irregular stromal interface is present but may need additional sections to identify.
- Well-developed lichen sclerosus is almost always present.
- Peripheral palisading is absent.

Pseudoglandular Carcinoma

- It is an unusual and aggressive variant of penile carcinoma with pseudoglands that are often filled with amorphous material.
- Also known as adenoid or acantholytic carcinoma
- Common sites involved are glans, coronal sulcus and inner foreskin.
- Higher metastatic rates and cancer-related deaths are seen when compared with usual SCC.
- The hallmark microscopic feature is the presence of **pseudoglands.**
- These are open spaces lined by atypical, cuboidal uni- or multistratified cells filled with amorphous material containing debris, keratin and desquamated cells.
- Frequent vascular and perineural invasions are seen.
- An important differential diagnosis is adenosquamous carcinoma**.**
- The important points of differentiation are as follows:
  - Presence of true glandular lining which is filled with mucin.
  - More homogeneous.
  - Squamous areas predominate.
  - Positive for CEA and mucin stains.

Verrucous Carcinoma

- It is a slow-growing, extremely well-differentiated variant with low malignant potential.
- Glans is the preferred site but there is occasionally extension to other compartments.
- It tends to be multi-centric in the foreskin.
- Microscopy shows a broad base and pushing borders.
- Dense inflammatory infiltrate may obscure tumor stroma boundary.
- Low Ki-67.
- Important differential diagnoses are **papillary carcinoma and Warty carcinomas.**

- The important points of differentiation with papillary carcinoma are as follows:
  - It has more atypia.
  - There are irregular but evident fibrovascular cores.
  - Jagged margins in contrast with the pushing margins in verrucous carcinoma.
- The important points of differentiation with warty carcinoma are as follows:
  - Warty carcinoma shows Koilocytotic change.
  - Jagged tumor margins.
  - Neoplastic cells show more pleomorphism and atypia.

Carcinoma Cuniculatum

- The word "cuniculous" means "rabbits burrow".
- It is a rare, low-grade variant of verruciform penile carcinoma with a deeply penetrating and burrowing pattern of growth.
- Elderly men are usually affected with a mean age of 77 years.
- Glans and coronal sulcus are the preferred sites.
- Tumours begin as small warts and slowly progress to larger tumors.
- Microscopy shows a verruciform pattern of growth.
- However, the tumor cells invade deep erectile tissues with a **burrowing pattern** of growth on the cut section.
- Cyst-like and sinus structures can also be observed.

Papillary Carcinoma

- It is a slow-growing, low-grade variant of squamous cell carcinoma.
- Glans, possibly with foreskin and coronal sulcus are the common sites.
- Microscopically, papillary structures with a fibrovascular core, mild to moderate acanthosis and hyperkeratosis are seen.

Adenosquamous Carcinoma

- The median age of these cases is 59 years.
- The various theories of origin given are as follows**:**
  - Origin in misplaced glandular cells in the perimeatal region.
  - Metaplastic goblet cells of foreskin mucosa.
  - Aberrant differentiation of squamous epithelium.
- Neoplastic squamous nests intermingled with areas of glandular differentiation are seen.

Sarcomatoid Carcinoma

- It is an aggressive variant of penile squamous cell carcinoma composed mostly of anaplastic spindle cells.
- Median age is 59 years.
- Glans is most commonly involved but the extension to coronal sulcus and foreskin can be seen.
- May occur after radiation therapy.
- There is a high mortality rate (40–75%).
- Inguinal nodal metastases are seen in 75–89% cases.
- Local recurrence is reported in 67% cases.

- Grossly, large gray-white or red polypoid or fungating mass with frequent ulceration and hemorrhage is seen
- Microscopy shows predominantly anaplastic spindle cells resembling fibrosarcoma or leiomyosarcoma.
- Occasional giant or multinucleated malignant fibrohistocytoma-like cells can be seen.
- Foci of usual squamous cell carcinoma may be present.
- There is prominent necrosis and mitotic activity.

UPDATED HISTOLOGICAL GRADING OF PENILE SQUAMOUS CELL CARCINOMA

- In the updated WHO classification (2016) of penile carcinoma, the tumors have been divided into three grades.

Grade 1

- These tumors show extreme differentiation.
- Keratinization and maturation are seen.
- Nuclear atypia is minimal or absent.
- Tumors grow in large nests.
- For example, verrucous, carcinoma cuniculatum, pseudohyperplastic, papillary, well-differenitiated usual SCC.

Grade 3

- The tumor cells are arranged in the form of solid sheets or trabeculae.
- There is scant keratinization and predominantly anaplastic cells are seen.
- There is no maturation.
- Cells are pleomorphic and show numerous mitoses.
- For example, sarcomatoid, basaloid, clear cell, lymphoepithelioma-like, pseudoglandular, poorly differentiated usual SCC.

Grade 2

- Intermediate between grade 1 and grade 3
- For example, warty carcinoma, moderately differentiated usual SCC.

PENILE INTRAEPITHELIAL NEOPLASIA

- In the updated WHO classification (2016) of penile carcinoma change has been made in Penile intraepithelial neoplasia (PeIN).
- They are now divided into three groups:
- **Non-HPV related PeIN:**
  - Differentiated (Simplex) PeIN
- **HPV-related PeIN:**
  - Basaloid PeIN
  - Warty PeIN
  - Warty basaloid PeIN
- **Other rare patterns of PeIN:**
  - Pleomorphic PeIN
  - Spindle PeIN

- Clear cell PeIN
- Pagetoid PeIN.

MOLECULAR CHANGES IN PENILE CARCINOMA

- HPV-positive cases have shown to have *MYC* amplification with integrated HPV DNA sequences.
- Conversely, HPV-negative tumors have been found to express significantly more **phosphorylated *EGFR*** than HPV-positive tumors with a corresponding increase in *pAkt* expression.
- Lymph node metastasis is associated with negative p16 expression as well as loss of heterozygosity and promoter hypermethylation of *p16INK4A.*
- Genomic alterations mapped at *3p* and *8p* were related to worse prognostic features including advanced T stage, recurrence, and death from disease.

Treatment

- Current literature suggests immediate and prophylactic inguinal lymph node dissection (ILND) carries improved survival rather than a delayed or therapeutic ILND.
- Current guidelines recommend systemic therapy for those with advanced nodal disease before consolidative surgery if responding to treatment, as well as for those exhibiting high-risk pathologic features.
- Current recommended systemic regimens in metastatic penile cancer are as follows**:**
  - Cisplatin-based regimens:
    - Paclitaxel, ifosfamide and cisplatin [TIP]
    - 5-FU and cisplatin.

POINTS TO REMEMBER

- The recent WHO 2016 classification divides squamous cell carcinoma as well as its precursor lesions into HPV related and non-HPV related.
- Three new entities have been added—warty basaloid, papillary basaloid and lymphoepithelioma like.
- p16 immunostaining has been suggested as a simple surrogate indicator of HPV.
- Each histological variant has been graded in a 3-tier grading system.
- Change has been made in penile intraepithelial neoplasia (PeIN).
- They are now divided into three groups, HPV related, non-HPV-related and other rare patterns.

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Immune Therapy Related Lymphoproliferative Disorders

24

INTRODUCTION

- A variety of therapeutic agents may increase the risk of lymphoproliferative disorders/neoplasms.
- Many of these are now recognized in the current WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues.
- These include broadly two categories of lesions:
  - Lymphoid neoplasms that arise after treatment for other malignancies and
  - Lymphoproliferative disorders/ neoplasms that develop in the context of therapy used to treat or prevent certain diseases (or graft rejection) that alter the immune system.

Post-Transplant Lymphoproliferative Disorders

- Post-transplant lymphoproliferative disorders (PTLDs) are lymphoid or plasmacytic proliferations that develop secondary to the immunosuppression.
- These are all recipients of solid organ transplantation (SOT) or stem cell allograft.
- The current WHO categories of PTLDs are outlined in Table 24.1.
- Most cases occur in the first year after SOT.
- Among adult patients, the frequency of developing PTLD largely corresponds with the intensity and/or composition of the immunosuppressive regimen.
- Certain drugs may modify the risk of PTLD.
- Calcineurin inhibitors increase the risk.
- Cyclosporine increases the risk mainly in higher doses (46.6 mg/kg/day).
- Tacrolimus increases the incidence of PTLD two- to fivefold, compared to cyclosporine.
- OKT3 (anti-CD3 monoclonal antibody), thymoglobulin and ATG (anti-thymocyte globulin) increases the incidence.
- IL-2 receptor inhibitor antibodies (daclizumab and basiliximab) and alemtuzumab (anti-CD52 antibody) do not increase the risk.
- There is conflicting data regarding mTOR inhibitors.
- Sirolimus therapy after kidney SOT has been reported to cause a twofold increase in PTLDs.
- Anti-metabolites (i.e. azathioprine and mycophenolate mofetil) are associated with a lower/not increased risk.

Table 24.1: Classification of post-transplant lymphoproliferative disorder (PTLD)

Main categories	*Subcategories*
Non-destructive post-transplant lymphoproliferative disorders	Plasmacytic hyperplasia Infectious mononucleosis-like lesion Florid follicular hyperplasia
Polymorphic PTLD	
Monomorphic PTLD	B-cell neoplasms DLBCL Burkitt lymphoma Plasma cell myeloma Plasmacytoma Others T-cell neoplasms Peripheral T-cell lymphoma Hepatosplenic T-cell lymphoma
Classic Hodgkin lymphoma type PTLD	Classic Hodgkin lymphoma

- Betalacept, a new selective costimulation inhibitor, has shown an increased incidence of PTLD versus cyclosporine-treated patients.
- Kidney SOT recipients have the lowest frequency (~1%).
- Liver and heart SOTs, an intermediate frequency (1–2%); and heart-lung/lung (5–10%).
- Intestinal SOTs, the highest frequency (up to 20%).
- The frequencies are much higher in children.
- The risks after peripheral blood, stem cell, and bone marrow (BM) allografts are also low (approximately 1%).
- Majority are associated with EBV infection.
- Non-EBV-associated cases are due to other viruses such as HTLV, human herpesvirus 8, cytomegalovirus, simian virus 40 and hepatitis C virus.
- Majority of PTLDs after SOT are of host origin (~90%).
- Whereas, the majority after BM allografts are of donor origin.

Non-destructive Post-transplant Lymphoproliferative Disorders

- These are defined as lymphoid proliferations in an allograft recipient characterized by architectural preservation of the involved tissue and an absence of features that would be diagnostic of a malignant lymphoma.
- Usually they form mass lesions.
- They must be distinguished from other entities.
- Since the histological picture may be quite non-specific, the diagnosis requires the formation of a mass lesion and/or significant EBV positivity.
- These lesions tend to occur at a younger age than the other PTLDs.
- They are often seen in children or in adult solid organ recipients who have not had a prior EBV infection.
- The sites most commonly involved include lymph nodes or tonsils and adenoids.

- They often regress spontaneously with a reduction in immunosuppression or may be successfully treated by surgical excision.
- However, IM-like lesions can be fatal.
- In some cases, polymorphic or monomorphic PTLD may follow one of the non-destructive type lesions.

Morphology

- PH is characterized by numerous plasma cells, small lymphocytes, and generally infrequent bland-appearing immunoblasts.
- IM PTLD has the typical morphological features of IM, with paracortical/interfollicular expansion and numerous immunoblasts in a background of T cells and plasma cells.
- Florid follicular hyperplasia is a mass lesion with marked follicular hyperplasia that does not suggest IM.
- Criteria for the distinction of these non-destructive PTLDs from other reactive lymphoid infiltrates are not well defined.
- Thus, the diagnosis rests on the extent of proliferation, clinical correlation and the presence or absence of EBV.

Immunophenotype and Genetics

- Immunophenotypic studies show an admixture of polytypic B cells, plasma cells, and T cells.
- EBV is present in many of the reported cases of PH and florid follicular hyperplasia.
- These diagnoses should be made only with great caution in EBV-negative cases, due to the non-specificity of the histological/immunophenotypic findings.
- IM PTLDs are typically EBV+ with EBV LMP1+ immunoblasts.
- Clonality studies usually reveal a polyclonal cell population.
- Although small monoclonal or oligoclonal populations may be observed.
- The significance of these findings is unknown.

Polymorphic Post-transplant Lymphoproliferative Disorders (P-PTLD)

- These are composed of a heterogeneous population of immunoblasts, plasma cells, and small and intermediate-sized lymphoid cells.
- They efface the architecture of lymph nodes or form destructive extranodal masses.
- However, they do not fulfill the criteria for any of the recognized types of lymphoma.
- Their distinction from monomorphic T cell PTLD is of great clinical importance but is not straightforward always.
- The reported frequency of P-PTLDs varies widely.
- However, in general, they are quite rare.
- The exception is the fact that in children P-PTLD is generally more common.
- It frequently follows post-transplantation primary EBV infection.
- The clinical presentation of P-PTLDs is not distinguishable from that of PTLDs in general.
- Reduction in immunosuppression leads to regression in a variable proportion of cases.
- Others may progress and require treatment for lymphoma.

Morphology

- In contrast to non-destructive PTLD lesions the effacement of the underlying tissue architecture is the key finding.
- However, unlike lymphomas they show the full range of B cell maturation, from immunoblasts to plasma cells, with small and medium-sized lymphocytes and cells with irregular nuclear contours.
- There may be areas of geographical necrosis and scattered large, bizarre cells.
- These cells may resemble Reed-Sternberg cells (atypical immunoblasts).
- Numerous mitoses may be present.
- Some cases have areas that appear more monomorphic.
- Thus, there may be a continuous spectrum between these lesions and M-PTLD.
- Other P-PTLDs have features that more closely resemble those of Hodgkin lymphoma.

Immunophenotype and Genetics

- Immunophenotypic studies demonstrate B cells and a variable proportion of heterogeneous T cells.
- Light chain class restriction does not exclude the diagnosis.
- When present, may be focal, or with the presence of different clonal populations.
- Prominent CD30 expression is common, but unlike in most cases of classic Hodgkin lymphoma, the CD30+Reed-Sternberg-like cells are CD20+ and CD15–.
- Most cases of P-PTLD contain numerous cells positive for EBV—encoded small RNA (EBER).
- Most cases demonstrate clonally rearranged IG genes.
- EBV terminal repeat analysis is the most sensitive method for demonstrating clonal populations in the EBV-positive cases.
- About 75% of P-PTLDs are reported to have mutated IGV genes without ongoing mutations, and the remainder is unmutated.
- Clonal cytogenetic abnormalities may be present.
- Comparative genomic hybridization studies also demonstrate abnormalities including BCL6 somatic hypermutations and aberrant promoter methylation.

MONOMORPHIC POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS (M-PTLD)

- M-PTLDs fulfil the criteria for either a B cell (i.e. diffuse large B cell lymphoma, Burkitt lymphoma, plasma cell myeloma, and plasmacytoma-like lesion) or T/NK-cell neoplasm (i.e. peripheral T cell lymphoma, NOS and hepatosplenic T cell lymphoma being the most common).
- B cell PTLD is the most common type of monomorphic PTLD.
- Genetically, the B cell type of monomorphic PTLD reveals a clonal IG gene rearrangement in virtually all cases.
- Majority contains EBV genomes in clonal episomal form.
- Cases of T cell origin demonstrate a clonal T cell receptor gene rearrangement.
- Alterations of c-MYC, N-RAS and p53 are infrequent—they occur in monomorphic PTLD and multiple myeloma, but not in polymorphic PTLD.
- Rearrangement of BCL-6 is also rare; however, its mutation is more frequent and carries a poor prognosis.
- Epigenetic alterations may also occur, such as the hypermethylation of O_6-methylguanine-DNA methyltransferase.

- It is a DNA repair gene.
- This abnormality is seen in 60% of monomorphic PTLD cases.
- Microsatellite instability and the defects of DNA mismatch repair occur more often in PTLD than in non-Hodgkin lymphomas of non-immunodeficient patients.
- Monomorphic PTLDs generally require therapy.
- They do not respond to reduce immunosuppression

CLASSIC HODGKIN LYMPHOMA TYPE POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDER

- Classic Hodgkin lymphoma (CHL)-type PTLD is the least common major category of PTLD.
- It usually occurs after kidney transplantation and is almost always EBV-positive.
- It demonstrates the classical morphological and immunophenotypic features of CHL.
- The mixed cellularity subtype is most frequent within this category.

ATYPICAL LYMPHOPROLIFERATIONS AND LYMPHOMAS ASSOCIATED WITH THERAPY FOR (AUTO) IMMUNE DISEASES

Methotrexate-associated Lymphoproliferations

- Methotrexate (MTX) has been used for a several decades, particularly for patients with rheumatoid arthritis.
- The mechanism of action as a chemotherapeutic agent is that it inhibits the enzyme dihydrofolate reductase.
- This decreases the production of thymidine (a pyrimidine), leading to impaired DNA synthesis and consequent cytotoxicity.
- In contrast, its action as an immunosuppressant is thought to be distinct from this mechanism and remains poorly understood.
- The effects are primarily on T cell function, including impairment of T cell activation, decreased expression of adhesion molecules by T cells, increased numbers of regulatory T cells, and a shift from Th1 to Th2 cells and cytokines.
- Of all lymphoproliferations arising in the setting of immunotherapy for an (auto) immune disease, the association is best known with the use of methotrexate (MTX).
- These lymphoproliferations and lymphomas have mostly, but not exclusively, been described in the setting of rheumatoid arthritis (RA).
- Typically, patients who develop lymphoma on MTX have been on long-term (median 3 years, sometimes up to 10 years) low-dose (5–25 mg/week) therapy.
- Other less common underlying disorders include psoriasis and dermatomyositis.
- The lymphomas seen with MTX are typically diffuse large B cell lymphomas (DLBCL) and classical Hodgkin lymphomas (CHL), as well as mimics of the latter.
- DLBCL and CHL are far more commonly associated with MTX therapy as compared with other immunodeficiency scenarios as well as those seen with the use of immunomodulatory agents.
- Other less commonly reported lymphomas in this setting are follicular lymphoma, Burkitt lymphoma, peripheral T cell lymphoma, lymphoplasmacytic lymphoma and small lymphocytic lymphoma.
- The frequency is not well documented; as there are only case reports.
- Approximately 40% are extranodal.

- One very interesting point is that they tend to be EBV-positive.
- The rest of the EBV-negative lymphomas, presumably unrelated to immunosuppressive therapy, actually account for the majority of lymphomas in RA patients.
- Both bona fide classical Hodgkin lymphoma (CHL) and Hodgkin-like lymphoproliferations (HLL) have been described in patients treated with MTX.
- It is essential to distinguish CHL from HLL.
- This is because the latter is much more likely to remit following the cessation of MTX therapy.
- The features that help in this distinction are presented in Table 24.2.

Table 24.2: Differences between classical Hodgkin lymphoma and its mimic Hodgkin-like lymphoproliferation

Feature site of disease	*CHL nodal > extranodal*	*HLL extranodal > nodal*
Morphology Hodgkin/ Reed-Sternberg cells background cells	Present Heterogeneous, lymphocytes are small and mature-appearing	Present, large and polyploid May be more homogeneous, lymphocytes may be immuno-blastic
Immunophenotype HRS cells CD30 CD15 CD20 CD45 Lymphocytes in the background	+ + –/+ – Mostly T cells	+ – + + Mostly B cells
EBV	In HRS cells only (40%)	In both 'HRS' cells (100%) and background (B) lymphocytes
IGH@ PCR	B cell clone rarely detected	B cell clone often detected

Anti-metabolite-associated Lymphoproliferations

- Studies suggest that the use of thiopurines like azathioprine and 6-mercaptopurine for the therapy of inflammatory bowel disease is associated with a 3–5 increased risk for the development of lymphoma.
- Many of these patients are EBV-positive.
- Mycophenolate mofetil in autoimmune disorders, such as systemic lupus erythematosus, has been associated with the development of EBV-positive DLBCL involving the central nervous system.

Immunomodulatory Agent-related Lymphoproliferations (IARLPDs)

- Several biologically targeted agents have recently been developed for the treatment of a variety of autoimmune disorders.
- These diseases include rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), psoriasis (PS), psoriatic arthritis (PA), multiple sclerosis (MS) and ankylosing spondylitis (AS).
- Their use and association with lymphoproliferations has been observed mostly in RA, IBD and PA.
- In contrast, lymphoproliferations are rather uncommon in the setting of MS, AS and PS.
- Within the IBDs, there is more of an association with Crohn's disease than there is with ulcerative colitis.

- In contrast to what has been observed in MTX-related lymphomas, many different types of lymphoma have been reported in association with immunomodulatory agents (IAs).
- These include a spectrum of both B cell and T cell lymphomas as well as NK-cell neoplasms and Hodgkin lymphoma.
- The epidemiology of IARLPDs is not well characterized and there are conflicting data in the published literature.
- Part of the reason is that they are quite uncommon and/or underrecognized.
- Regression is more likely to occur in the context of EBV-positivity.
- In general, however, this favorable response to therapy withdrawal is more likely to occur in the setting of MTX than it is with IAs.
- The specific lymphoma type might also determine whether responses occur.
- For example, hepatosplenic T cell lymphomas are usually rapidly fatal, regardless of cessation of immunomodulatory treatment.
- In general, in the setting of IAs, polymorphic lymphoproliferations are less common than overt lymphomas, accounting for approximately 10% and 90%, respectively.
- A spectrum of different lymphoma types has been reported, dominated by DLBCL (45%) and CHL (20%).
- Follicular lymphoma (5–10%) and a spectrum of T cell lymphomas (5%) are also seen.
- Uncommon miscellaneous subtypes include Burkitt lymphoma, MALT lymphoma, lymphoplasmacytic lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma and NK-cell neoplasms.
- There is also a variable association with EBV amongst these different subtypes.
- This suggests a somewhat different pathophysiology, and that this oncovirus might not be involved in the initiation of IARLPDs.
- Within specific lymphoma subtypes, EBV is most often seen in CHL (80%) and less so in DLBCL (25%).
- Interestingly, up to 25% of follicular lymphomas may be EBV-positive.
- Although the causal relationship between IAs and lymphoma has been questioned by many authors.
- It is difficult to ignore the noted temporal associations, with both the short latency to develop and brief time to regress.

Hepatosplenic T cell Lymphoma

- It is a specific lymphoma subtype that is tightly linked to the therapy of one group of immune-mediated diseases.
- The association of hepatosplenic T cell lymphoma (HSTCL) is with IBD.
- It is more so with Crohn's disease (CD) than ulcerative colitis (UC).
- HSTCL is a rare and highly aggressive form of T cell lymphoma, comprising 5% of all T cell lymphomas and ~1% of lymphomas overall.
- The available data more compellingly implicate thiopurines, as compared with anti-TNF-α therapy, with the development of HSTCL.
- However, it is currently unclear to what degree the use of such biological therapy adds to the risk imparted by thiopurines.
- Risk factors associated with the development of HSTCL in IBD appear to be young age (between 10 and 35 years) and male gender (men are 10 as likely as women to develop HSTCL).
- Thus, young men in particular should be monitored with enhanced vigilance.

POINTS TO REMEMBER

- A variety of therapeutic agents may increase the risk of lymphoproliferative disorders/neoplasms.
- These include those agents used to treat other malignancies (i.e. cytotoxic chemotherapy) and those used to treat or prevent certain diseases (or graft rejection) that alter the immune system.
- Post-transplant lymphoproliferative disorders and those lymphoproliferations associated with the therapy of autoimmune and other immune-mediated diseases.
- PTLD is more common after a solid organ transplant.
- The most common types are DLBCL and CHL.
- Three drugs, or classes of drugs, used in the treatment of autoimmune and other immune-mediated diseases including methotrexate, anti-metabolites (including thiopurines and mycophenolate mofetil), and immunomodulators.
- They have been implicated in causing an increased risk of acquiring lymphoma.
- The appropriate recognition of these disorders is important to correctly classify and institute appropriate therapy.
- It is important to recognize that reduced immunosuppression or withdrawal of therapy may be necessary, rather than treating as a malignant lymphoma.

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Section

3

Hematology

AML Transcriptomics and Proteomics

25

Poorvi Kapoor

INTRODUCTION

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal disorder characterized by the accumulation of acquired somatic genetic alterations in hematopoietic progenitor cells that alter the normal mechanisms of self-renewal, proliferation and differentiation.

- Approximately 55% of adults with AML show non-random clonal chromosomal aberrations which include the following:
  - Balanced translocations
  - Inversions
  - Deletions
  - Monosomies
  - Trisomies.

WHO Classification (2016)

- Acute myeloid leukemia with recurrent genetic abnormalities
  - AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1
  - AML with inv(16)(p13.1q22) or
  - Acute promyelocytic leukemia with PML-RARA
  - AML with t(9; 11)(p21.3;q23.3); KMT 2A-MLL T3
  - AML with t(6;9)(p23;q34.3); DEK-NUP214
  - AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); RBM15-MKL1
  - AML with BCR-ABL 1
  - AML with mutated NPM1
  - AML with biallelic mutation of CEBPA
  - AML with mutated RUN X1
- AML with myelodysplasia-related changes
- Therapy-related myeloid neoplasms
- Acute myeloid leukemia, not otherwise specified
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukemia
  - Acute monoblastic/monocytic leukemia

- Pure erythroid leukemia
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

- Myeloid sarcoma
- Myeloid proliferations associated with Down syndrome
  - Transient abnormal myelopoiesis associated with Down syndrome
  - Myeloid leukemia associated with Down syndrome
- Blastic plasmacytoid dendritic cell neoplasm.

Molecular Pathogenesis of AML

- There are a few fundamental features of AML:
  - Inappropriate proliferation in the absence of normal growth signals
  - Escape from programmed cell death
  - Block of differentiation
  - Aberrant cell cycle control
  - Multiorgan dissemination of leukaemic cells
  - A single mutation is not enough to cause AML
  - Germline mutations predispose to leukemia, leukemia phenotype occurs following additional somatic mutation.
- **Types of mutations:**
  - *Class I:* These mutations activate signal transduction pathways and enhance proliferation and/or survival, e.g. activation of receptor tyrosine kinase FLT3, RAS signaling pathway (Table 25.1).
  - *Class II:* Affect transcription factors or components of the transcriptional co-activation complex leading to impaired differentiation and/or aberrant acquisition of self-renewal properties, e.g. t(8;21), inv(16), t(15;17), CEBPA, MLL.

Table 25.1: Mutations with the genes involved and prognostic implications

Chromosomal aberration	*Genes*	*Prognosis*
t(8;21)	RUNX1-RUNX1T1	Good
inv(16) or t(16;16)	CBFB-MYH11	Good
t(15;17)	PML-RARA	Good
t(9;11)	MLLT3-MLL	Intermediate
t(6;9)	DEK-NUP214	Poor
inv(3) or t(3;3)	RPN-EVI1	Worst
t(1;22)	RBM15-MKL1	Good
Rearrangements of MLL gene	MLL	Poor
Monosomy 7 or del 7q31	—	Poor
Deletion of 5q31	?	Unknown
Complex chromosomal aberrations	?	Worst

Molecular Pathology of AML

- Abnormal cell proliferation
  - FLT3 mutation

- RAS mutation
- C-KIT mutations

- Block in differentiation
  - CBF AML t(8;21) and inv(16)
  - PML-RARA t(15;17)
  - MLL translocations
  - Hox gene translocations
  - CEBPA mutations
- Suppression of apoptosis
  - BCL2 overexpression
- Capacity for indefinite cell renewal.

Mutations Blocking Differentiation

- Core binding factor-related AML
  - Core binding factors (CBFs) are heterodimeric complexes (CBF-α and CBF-β) that regulate expression of genes involved in hematopoietic cell differentiation.
  - AML with t(8;21) and AML with inv(16)/t(16;16) show this mutation.
  - They are often grouped based on the involvement of related CBF machinery and have a relatively favorable prognosis.
  - RUNX1/AML1 binds to the DNA promoter sequences of genes needed for hematopoiesis and CBF-β protects the complex from proteolysis (Fig. 25.1).

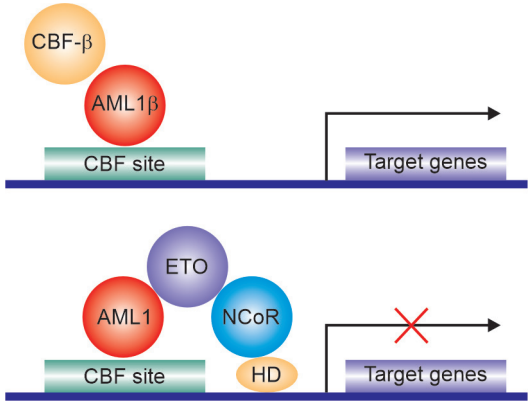

Fig. 25.1: Mechanism of CBF blocking stem cell differentiation

- AML with inv(16)/ t(16;16)
  - Translocation and/or molecular fusion of CBFB and MYH11
  - The fusion gene acts as a dominant inhibitor of CBF
  - This causes suppression of transcription of genes crucial for hematopoiesis and differentiation
  - It morphologically encompasses FAB M4Eo category and shows abnormal eosinophils.
- AML with t(15;17)
  - The retinoic acid receptor α is involved in the transcription of genes necessary for differentiation.
  - t(15;17)(q22;q22) causes the formation of PML-RARα fusion gene.

- Other translocations are also known to occur and form different fusion genes like t(11;17) forms PLZF-RARα and t(5;17) forms NPM-RARα.
- PML-RARα is a transcriptional repressor and recruits the nuclear co-repressor complex and histone deacetylases (Fig. 25.2).

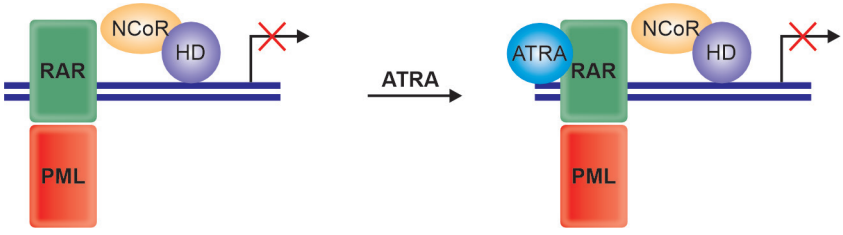

Fig. 25.2: Mechanism of action of all-trans-retinoic acid (ATRA) on PML-RARα fusion gene

- AML with t(9;11)(p22;q23); MLLT3-MLL
  - These mutations are responsible for around 9–11% pediatric AML and about 2% AML in adults
  - They show monocytic morphology and +8 most common additional anomaly
- Mixed lineage leukemia (MLL) gene
  - MLL is a histone methyltransferase that regulates Hox expression and Hox family members play an important role in gene transcription by acting as a positive regulator.
  - Normal MLL requires cleavage by Caspases into its mature form, which thereby dysregulates gene expression and causes a block in differentiation.

Mutations Causing Excess Proliferation

- **FLT3:**
  - It is a receptor tyrosine kinase expressed in 70–100% of AML and activating mutations of FLT3 are seen in 30% of AML cases
  - Activating mutations like internal tandem duplications and missense point mutations in the tyrosine kinase domain are most frequently seen and these lead to deregulated proliferation of AML cells
  - Mutated FLT3 is not included as a separate entity as it is frequently associated with other entities as well but should be mandatorily performed on all cytogenetically normal patients.
  - FLT3-ITD is known to confer a poor prognosis and overall survival.
- **AML with mutations of NPM1:**
  - Protein nucleophosmin acts as a shuttle for other proteins between the nucleus and the cytoplasm
  - It also plays a role in cell cycle control and the regulation of centromere duplication for facilitating orderly mitosis
  - 50% cases of AML which are karyotypically normal show NPM1 mutations while 90% of AML with monocytic differentiation show this mutation
  - NPM1 mutations cause an abnormal accumulation of proteins in the cytoplasm
  - Mutations in NPM1 have a favorable prognosis especially when FLT3 mutations are absent.

- **AML with CEBPA mutations**
  - CEBPA encodes a transcription factor essential for granulocytic differentiation
  - Mutations cause interference with this protein's contribution to terminal differentiation
  - These mutations are less common than NPM1 and are only seen in around 20% of cytogenetically normal cases and is most commonly seen in AML M0 or M1 cases
  - Isolated CEBPA mutations confer a good prognosis.

Transcriptomics

- The study of the complete set of RNAs (transcriptome) encoded by the genome of a specific cell or organism at a specific time or under a specific set of conditions.
- **Need for transcriptomics:**
  - Among AML with relatively good prognosis and abnormal cytogenetics are t(8;21), t(15;17) and inv(16) while there are other cytogenetic abnormalities which define a poor prognostic group.
  - However, a majority of AMLs are cytogenetically normal and collectively define an intermediate prognostic group.
  - Thus the study of transcriptomics is essential for identifying different prognostic groups.
  - RNA profiling provides clues to the functional differences between tissue and cell types, function and interaction between genes, gene regulation and regulatory sequences, as well identification of candidate genes for any given disease.
- **Basic steps of gene expression:**
  - DNA consists of exon and introns, which undergoes transcription to form Pre-mRNA (Table 25.2), which further undergoes modification like addition of a cap at the 5′ end, a polyadenylated tail at the 3′ end and RNA splicing, which collectively come under post-transcriptional modification.
  - After the splicing occurs, the ends of exons are joined which now forms RNA and are transported outside the nucleus and translated into the cytoplasm to form proteins.
  - Alternative splicing adds to the complexity of the genome.

Table 25.2: Types of RNA and their characteristics

Type	*Name*	*Number*	*Function*
mRNA	Messenger	~30000	Coding for proteins
rRNA	Ribosomal	~300–400	Component of ribosome
tRNA	Transfer	~1000	Translation
snRNA	Small nuclear	?	Splicing
snoRNA	Small nucleolar	~375	RNA modification
miRNA	microRNA	~1000	Gene expression regulation
piRNA	piwiRNA	~33000	Gametogenesis, transposon defense
lncRNA	Long non-coding	~35000	Regulation, imprinting

- **ENCODE project:**
  - The National Human Genome Research Institute (NHGRI) launched a public research consortium named **ENCODE**, the Encyclopaedia Of DNA Elements, in 2003, to carry out a project to identify all functional elements in the human genome sequence

- The Pilot phase (2003–2007) was where there was the identification of all functional elements in 1% of the human genome while the Production phase (2007 onward) identified all functional elements in the whole genome
- They found that there was a pervasive transcription of the human genome, identified many novel non-protein transcripts as well as numerous transcription start sites and discovered that there were multiple overlapping transcripts of different sizes from the same/opposite DNA strands.

Advantages and Uses of Transcriptomics

- Explores unknown players in a wide range of biological processes, diseases, traits and responses to stimuli.
- It is the first step to implicate novel genes and pathways that may be involved or associated with a particular condition.
- However, a difference in expression per se is not sufficient evidence to infer a direct involvement of the gene in a particular process or trait.
- Discovery of markers helps identify a therapeutic target, is of prognostic significance and helps in a better understanding of the pathogenesis.
- The expression of thousands of genes is measured simultaneously.
- It provides a snapshot of all genes that are actively transcribed during a particular process.
- Upon comparing these measurements, it identifies those genes that are expressed at higher or lower level under a particular condition.

Methods for Transcriptome Analysis

- **Gene expression arrays:**
  - Quantification of transcript abundance
  - Single/multiple 3′ probes
- **Genome tiling arrays**
  - Identification of all transcribed sequences
  - Multiple probes along the genome
    - Alternative splicing arrays
  - Quantification of different RNA isoforms
  - Probes in exons and exon-exon junctions
- Microarrays
  - They are the most widely used technological platform for the whole genome sequencing, although the sequencing of the transcriptome is rapidly increasing in popularity
  - These are solid based platforms (e.g. glass slides) containing millions of copies for thousands of "reporter probes"
  - Reporter probes comprise a part of the sequence of the genes in the genome
- Procedure
  - Fluorescent labeled copies of the original mRNA are bound to the probes
  - The label intensities for each position on the array are measured
  - These positions are associated to their specific reporter probes
  - The presence and abundance of each transcript in the labeled RNA pool are then inferred
  - The advantage of microarray is that the technique is relatively cheap and the tools to analyze the data that have been developed, matured and tested

- **Expression arrays:** They are reported only on known or predicted genes
- **Tiling arrays:** They help identify novel transcripts, and non-coding RNA genes and have a better characterization of splice variants and exons and contain reporter probes across the full genome.

Transcriptome Sequencing

- It is more advanced than microarray.
- From the mRNA pool, the RNA is fragmented and primed and cDNA is synthesized, which is further amplified using the PCR method and then used as a template for high-throughput sequencing.
- The generated sequence information is mapped or assembled into a reference transcriptome, and the number of sequence copies generated for each gene is used to infer the number of mRNA copies in the original sample.
- **Advantages:**
  - Sequencing approaches provide more comprehensive information on the transcript characteristics like splice variants, mRNA sequence variations and gene fusions.
  - They are not limited to the known or predicted genes of an organism or the genes represented on a microarray.
- **Disadvantages:**
  - Quality control, pre-processing and analysis procedures have not yet been fully matured.
  - The assembly of, or mapping against, the reference transcriptome requires substantial computing power.

Transcriptomics of AML

- A meta-analysis of 25 gene expression studies examined 4918 genes and observed the upregulated and downregulated genes in acute leukemia with their frequencies of expression.
- The top-ranked up-regulated genes associated with poor prognosis were BCL11A, TBXAS1, HOXB5, CD34 and RBPMS.
- The top-ranked down-regulated genes associated with poor prognosis were EML4, C3AR1, SMG1, FOXO1 and IL6ST.

Proteomics

- It is the science that focuses on the study of proteins including their role, localization, structure and interactions.
- There are three main components of expressional proteomics
  1. Protein separation
  2. Analysis
  3. Bioinformatics
- **Sample preparation:**
  - Sample can be taken from organisms as a whole or tissue, fluids and cells.
  - It typically involves solubilization, denaturation and reduction and treatment of sample proteins.

- It is important to prevent cell lysis from occurring as this may lead to the release of proteases causing proteolysis and therefore, protease inhibitors are added to minimize artifactual proteolysis.
- Some examples of these inhibitors are phenyl methyl sulphonyl fluoride (PMSF), EDTA and ethylene glycol tetraacetic acid (EGTA) and sample processing in cold conditions also aid in reducing proteolysis.
- Protein separation depends on certain specific protein biophysical parameters like isoelectric point, molecular weight and affinity.

- **Methods:**
  - Chromatographic methods like HPLC and 2D-HPLC
  - Electrophoretic methods like SDS-PAGE and 2D electrophoresis
  - Reverse phase (RPLC) hydrophobicity.
- **Sample separation:**
  - 2 major approaches are used to separate the sample—gel-based and gel-free approach
  - 2D PAGE (polyacrylamide gel electrophoresis) is the centerpiece of gel-based separation method
  - Reverse phase resin (RPLC) separates compounds based on their hydrophobicity.
  - Multidimensional separation is used to address high sample complexity and combines several separation techniques coupled to improve the resolving power and each dimension uses different molecular properties as a basis for separation.

2D SDS PAGE

- This method separates proteins based on their isoelectric point, which is the pH at which the protein has no net charge.
- There are two dimensions of which the first is separation by isoelectric focusing.
- It is done with an immobilized pH gradient gel strip containing a low concentration of polyacrylamide.
- Ampholytes are added to create a pH gradient in an electric field.
- The IEF gel with protein mixture is placed in an electrophoresis system for up to 24 hours and the proteins form tight bands at their isoelectric point and this is now ready for the second method.
- The second dimension causes separation by mass and has two parts, the stacking gel, which concentrates the sample and the running gel which is used to separate the proteins.
- The pH gel strip is loaded onto a SDS (sodium dodecyl sulphate) gel, which is used to mainly denature and linearize the protein.
- The IEF gel (from the first dimension), is put in one long well in the stacking gel and the proteins are subjected to an electrical field to separate.
- The larger proteins are found at the top and the smaller ones are at the bottom of the gel.
- **Disadvantages**
  - Lacks reliable reproduction and has low precision
  - Slow and tedious with frequent "open" phases where contamination is possible
  - Not for hydrophobic proteins
  - Limited by pH range
  - Not easy for low-abundant proteins.

Mass Spectrometry

- It allows compounds to be identified by the gas phase production of ions and their subsequent separation and detection based on their mass-to-charge ratio (m/z).
- It has a high level of specificity and sensitivity.
- **Principle components:**
  - Ionization source
  - Mass analyzer
  - Detector
  - Computer.
- It relies on molecules being converted into ions in the gas-phase.
- Two ionization techniques are present:
  - Electrospray ionization
  - Matrix-assisted laser desorption ionization (MALDI)
  - Both these approaches are known as **soft ionization techniques**, allowing molecules to remain relatively intact during the ionization processes.

MALDI

- **Principle:**
  - The protein (analyte) is mixed with a large excess of matrix, which is a small molecule organic material in aqueous/organic solvent.
  - It helps dilute analyte preventing clusters or aggregates too large to desorb.
  - Typical matrix materials are small, highly conjugated organic acids like 2,5-dihydroxybenzoic acid (DHB).
  - Analyte and matrix solution is mixed and a few microliter is added to the MALDI target plate, which evaporates leaving behind a crystallized mix.
  - The target plate is put in the high vacuum source of the mass spectrometer and irradiated with a pulse laser beam of 337 nm.
  - The matrix absorbs most of the energy as heat and the protein remains intact.
  - A dense gas cloud is formed by sublimation of the matrix and analyte, which rapidly expands into the gas phase.
  - This heads supersonically toward the vacuum and energy is transferred from matrix into the analyte, which gets protonated/deprotonated due to collisions between the neutral analyte and excited matrix ions.
  - These analyzer molecules move toward the mass analyzer and are detected using electron multiplier or array detectors.
  - Ions are separated based on their mass-to-charge ratio.
  - The ions are detected after mass analysis by converting the detector-surface collision energy of the ions into emitted ions, electrons, or photons that are then sensed with various light or charge detectors.
  - Examples are quadrupoles, ion-traps and time of flight instruments.
- **Time of flight mass analyzers:**
  - It is one of the simplest mass analyzers used in mass spectrometry and is often interfaced with MALDI ionization source.
  - It is extremely rapid and offers high resolution and sensitivity and is a popular choice in techniques such as proteomics.
  - All the ions are accelerated with the same potential, i.e. all have the same kinetic energy.

- Singly charged ions with a larger mass will travel with a lower velocity down the tube compared to those with a smaller mass, i.e. they separate according to their velocities.
- It is this simple feature of TOF analyzers that is conducive to their large mass range, making it ideal for coupling with MALDI for the analysis of large intact biomolecules such as proteins.

- There are 2 methodologies used for protein identification using mass spectrometry:
  - Peptide mass mapping where proteins are separated by electrophoresis, stained and excised for subsequent digestion and later subject to analysis by MALDI-TOF.
  - Peptide sequencing where peptides are collided with gas molecules inside the mass spectrometer causing fragmentation along the polypeptide backbone, which is mainly into 2 fragments b-ions and y-ions.
  - The resulting ion spectra produced can be compared with available amino acid sequences obtained from a protein database.
- Limitations of data analysis:
  - It is dependent on well-annotated genome databases.
  - The spectra are not always perfect.
  - Database searches only give scores, so the false positives have to be manually validated.

Proteomics in AML

- Proteins identified as specific targets of t(8;21)
  - Sorcin
  - Fuse binding protein 2
- Proteins identified as specific targets of AML patients with a normal karyotype
  - Nuclear receptor ROR alpha
  - Growth arrest-specific 6
- Proteins identified as specific targets of inv(16)
  - Nucleolar transcription factor 1 (UBF1)
  - **Transcription factor Mafk**
  - Translational control tumor protein
- Proteins identified as specific targets of complex aberrant karyotype
  - Hepatoma-derived growth factor
  - hnRNP Do
  - **B cell CLL lymphoma/leukemia 11A**
- Proteins identified as specific targets of 11q23 translocations
  - Stress-induced phosphoprotein 1.
- In some studies, certain proteins were identified that have not previously been reported in the context of AML cytogenetics.
- Casein kinase 1 alpha and RNA binding protein hnRNPA2/B1—in normal karyotype AML patients showed high and specific expression.
- Differential expression of cell cycle protein prohibitin in t(8;21) and inv(16) patients was seen.
- An apoptosis inhibitor protein (IAP) survivin identified as a specific target of t(8;21) AML was seen to be associated with advanced disease and poorer prognosis.
- Survivin is known to be highly expressed in most tumor cell types and absent in normal cells.

Therapeutic Potential

- Small interfering RNA (siRNA) are synthetic antisense oligonucleotides to the mRNA of the gene of interest that works to silence the expression of a particular gene by its complementary binding.
- siRNAs, such as LY2181308, bind to the respective mRNA which results in disruption of translation of that particular gene.
- The use of antisense siRNA survivin therapy hopes to render cancer cells susceptible to apoptosis by eliminating survivin expression in the cancer cells.

Cytogenetic Groups Differ in their Protein Interaction Networks

- Proteins identified by mass spectroscopy screening from different AML cytogenetic groups can be used to generate a wider protein interaction map.
- Ingenuity pathway finder software program provides the protein–protein interaction maps based on known literature that has been sited.
- Proteins matched to this database to generate protein–protein interaction maps (PPI).
- The major regulating network and the signaling pathways contributed by these networks are:
  - MAPK8 and MYC for complex aberrant karyotype
  - JUN and MYC for Inv(16)
  - TP53 for t(8;21)
  - TP53, MYC, PRKAC (cAMP dependant kinase) for 11q23.

POINTS TO REMEMBER

- Proteomics and transcriptomics approach is the future of studying leukemogenesis.
- There is a huge volume of data and a need for standardized software for analysis.
- It has diagnostic, prognostic as well as therapeutic significance.

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Aplastic Anemia: Recent Concepts

26

Poorvi Kapoor

INTRODUCTION

- It is a syndrome of bone marrow failure characterized by peripheral pancytopenia and marrow hypoplasia due to diminished or absent hematopoietic precursors in the bone marrow, most often due to injury of the pluripotent stem cell.
- There should be the absence of an abnormal infiltrate with no increase in reticulin.
- The designation of aplastic anemia is a misnomer because the disorder is defined as a pancytopenia instead of an anemia.

History

- It was first recognized by Dr Paul Ehrlich in 1888 in a young pregnant woman who died of severe anemia and neutropenia and the autopsy examination showed a fatty marrow with no hematopoiesis.
- The name aplastic anemia was applied to this disease in 1904 by Chauffard.
- It is derived from the Greek word "a" and "plasso" meaning 'without form'.

Epidemiology

- Aplastic anemia (AA) is a life-threatening bone marrow failure disorder if untreated and is associated with very high mortality.
- Bimodal age involvement is seen, occurring between 15 to 25 years and >60 years.
- There is no sex predilection seen.
- The incidence of aplastic anemia in the West is 2/million per year and is about 2 to 3 fold higher in Asia.
- The precise incidence of AA in India is not known due to lack of any epidemiological study.
- However, in a hospital-based study performed by Varma et al, they found that 20–40% of pancytopenic patients are diagnosed as AA in referral centers.
- In another epidemiological study performed in children in and around Lucknow, UP, showed the incidence of around 6.8 cases per million population per year.

Classification

- It is broadly classified into congenital and acquired.

- **Congenital**
  - Fanconi's anemia
  - Congenital dyskeratosis
  - Diamond-Shwachman syndrome.
- **Acquired**
  - Idiopathic
  - Secondary.
- Inherited AA presents earlier and is characterized by associated physical abnormalities and does not respond to immunosuppressants.
- It should be suspected in first-time diagnosis of AA.

Associations of Aplastic Anemia

- Aplastic anemia is known to be associated with:
  - Pregnancy
  - Viruses
  - Hepatitis
  - Pesticides
  - Benzene
  - Paroxysmal nocturnal hemoglobinuria
  - Certain drugs.

Clinical Presentation

- Commonly, they present with recurrent infections due to profound neutropenia.
- Infections are usually bacterial.
- Mucosal hemorrhages and other bleeding manifestations due to thrombocytopenia.
- It may occasionally present with fatigue and other symptoms due to progressive anemia.
- Fever
- Invasive fungal infection due to severe and prolonged neutropenia.
- Menorrhagia may also be seen in premenopausal women.

Approach to Diagnosis of Aplastic Anemia

- Careful history should be taken for intake of drugs, chemical exposure, infections or radiation exposure.
- Family history should be asked for bone marrow failure syndromes and early-onset of cancers.
- Physical examination should be thorough and congenital abnormalities, skin pigmentation and nail abnormalities should be looked for

Lab Investigations

- Complete blood count with peripheral smear examination (pancytopenia)
- Reticulocyte count, which is low
- Bone marrow aspirate
- Bone marrow biopsy (for diagnostic purposes)
- Cytogenetics

- Aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase and bilirubin
- Serological testing for HIV, EBV and hepatitis viruses
- Flow cytometry for paroxysmal nocturnal haemoglobinuria
- Chromosomal breakage studies for Fanconi's anemia
- Histocompatibility testing, needed for subsequent grafting of bone marrow

Diagnostic Criteria

- The criteria have been laid down by International Agranulocytosis and Aplastic Anemia Study Group, 1987 and are as follows:
  - Hemoglobin <10 g/dL
  - Platelet count <50,000/μL
  - Neutrophil count <1500 cells/ μL with a hypocellular bone marrow without infiltration or fibrosis.
- Severity criteria laid down by Camitta et al in 1976.
- **Severe:**
  - If marrow cellularity <25% for age and at least 2 of the following criteria
    - Neutrophil count $<0.5 \times 10^9$/L
    - Platelet count $<20 \times 10^9$/L
    - Absolute reticulocyte count $<20 \times 10^9$/L
- **Very severe:**
  - Above criteria are met and neutrophil count $<0.2 \times 10^9$/L.

Pathophysiology

- The responsiveness of aplastic anemia to immunosuppressive therapy is the best evidence of an underlying immune response.
- A non-immune pathophysiology has been inferred due to a failure of response to immunosuppression.
- Refractoriness to suppression is consistent with a very severe stem cell depletion, a "spent" immune response or immunologic mechanisms that are not susceptible to current therapies.
- Experiments have shown that lymphocytes from AA patients inhibit allogeneic and autologous hematopoietic colony formation *in vitro*.
- Cytotoxic T lymphocytes are the ones responsible for this destruction and overproduction of TNF and IFN-γ which are direct inhibitors of hematopoiesis and upregulate Fas on CD34+ cells.
- These immortalized CD4+ and CD8+ cells also secrete Th1 cytokines which are directly toxic to CD34+ cells.
- A reduction in the hematopoietic stem or progenitor cell is a universal finding in all aplastic anemia cases.
- There is an oligoclonal expansion of CD8+ CD28- T cells.
- Exposure to specific environmental agents, diverse host genetic risk factors, and individual differences in the characteristics of the immune response account for the disease's infrequency, variations in its clinical behavior, and patterns of responsiveness to treatment.
- Microarray of the scant CD34+ cells from marrow failure patients revealed a transcriptome in which genes involved in apoptosis, cell death, and immune regulation were up-regulated.

Detection of Stem Cells and T Cells

- Quantitative testing of CD34+ cells can be done by flow cytometric analysis.
- Qualitative testing can be done by evaluating the number of colonies derived from committed progenitors in semisolid media.
- The oligoclonal expansion of CD8+ cells can be assessed by:
  - Flow cytometric analysis for T cell receptor (TCR) Vβ subfamilies
  - Spectratyping to detect skewing of CDR3 (complementarity determining region) length
  - Sequencing of the CDR3 region to establish a molecular clonotype.
- These oligoclonally expanded Vβ subfamilies, diminish or disappear with successful therapy and original clones re-emerge with relapse.
- Not only a murine version of ATG and cyclosporine but also monoclonal antibodies to γ-interferon and tumor necrosis factor abrogated hematological disease

Genetic Basis for T cell Activation in Aplastic Anemia

- There is over-representation of HLA-DR2.
- Polymorphisms in cytokine genes—TNF-alpha, gamma interferon and interleukin-6 occur.
- There is a constitutive expression of T-bet, a transcriptional regulator that is critical to Th1 polarization.
- Mutations in PRF1, the gene for perforin (perforin is overexpressed).
- There are mutations in SAP, a gene encoding for a small modulator protein that inhibits γ-interferon production (SAP protein levels are markedly diminished).
- White blood cells in aplastic anemia have short telomeres (1/3 patients) in patients with dyskeratosis congenita.
- Inherited mutations in genes that repair or protect telomeres are genetic risk factors in acquired aplastic anemia.
- Certain genes like TERF1 and TERF2 gene products bind and protect telomerase.
- TERC and TERT mutations can occur in patients with acquired AA and also in family members of dyskeratosis congenita patients.
- Aplastic anemia may co-exist or appear to evolve into other hematological diseases (like PNH, MDS and acute leukemia) that are characterized by the proliferation of distinctive cell clones.
- However, the mechanisms linking immune-mediated and premalignant pathologies are unclear.
- Tiny clones can be picked up on flowcytometry or FISH.

PNH and Aplastic Anemia

- Approximately 50% of patients at presentation with pancytopenia have expanded populations of PNH cells detected by flow cytometry (the absence of glycosylphosphatidyl inositol-linked membrane proteins).
- The clones are small, and there is no thrombosis or hemolysis.
- PNH clones in aplastic anemia patients have survival benefits as compared to the normal target cells as they "escape" immune attack due to the absence of large numbers of cell surface proteins.

- These mutant clones proliferate and do not overexpress Fas.
- Residual cells of normal phenotype in PNH bone marrow up-regulate the same apoptosis and cell-death genes as do CD34+ cells in aplastic marrow.

Myelodysplasia and Aplastic Anemia

- Aneuploidy develops in a minority of patients over time and monosomy 7 or trisomy 8 is the most characteristic.
- Trisomy 8 is MDS with many immune abnormalities that resembles aplastic anemia.
- These patients respond to immunosuppressive therapies and oligoclonal T cell expansions are usually present in these cases.
- WT1 antigen, which is expressed by these aneuploid cells, gets recognized by immune cells, i.e. the oligoclonal T cells.
- But, there is no immune-mediated destruction of the target cells due to an associated up-regulation of certain anti-apoptotic like c-myc, survivin and CDK1.
- Monosomy 7 clones expand in an abnormal cytokine milieu.
- High G-CSF concentrations lead to a selection of cells that bear a short isoform of the G-CSF receptor that signals proliferation but not differentiation.

Treatment and Management

- The treatment of severe aplastic anemia, has improved dramatically over the last 25 years and long-term survival of more than 75% of patients can be anticipated.
- There are two main methods:
  1. Bone marrow transplantation
  2. Immunosuppression.
- Hematopoietic stem cell transplantation is indicated in:
  - Severe AA
  - Less than 40 years of age
  - Failure to respond to 1 course of ATG in children and 2 rounds in adults, who lack an HLA-compatible sibling (in some countries)
  - In current practice, unrelated transplant is offered for children who have failed a single course of immunosuppression and to adults who are refractory to multiple courses of ATG and alternative therapies such as androgens.
- Anti-thymocyte globulin (ATG) with Cyclosporin A (CsA)—indicated as first-line therapy for non-severe AA patients who are:
  - Transfusion dependent
  - Severe AA (SAA) patients >40 years of age
  - SAA patients <40 years who lack an HLA-identical sibling donor.
- **Anti-thymocyte globulin:**
  - It is an infusion of horse, rabbit, or rarely goat-derived antibodies directed against human T cells
  - It is a mixture of antibodies (polyclonal) intended to bind various cell surface antigens.
  - It has selective CD8 positive T cell suppressive action—complement-mediated lysis and T cell activation and apoptosis.

- It is both immunomodulatory and cytotoxic.
- It acts by modulation of surface molecules involved in endothelial leucocyte interaction, interference with dendritic cell function and induction of regulatory T cell and natural killer T cell.
- Response to ATG occurs in around 50% by 3 months and 70% to 75% by 6 months.
- Lack of response may be because of inadequate immunosuppression, irreversible stem cell deficiency (severe or very severe AA), or non-immune-mediated AA.

- **Cyclosporin A:**
  - It causes direct inhibition of the expression of nuclear regulatory proteins, resulting in reduced T cell proliferation and activation
  - It is less effective than either ATG alone or ATG plus cyclosporine
  - As with ATG, doses and length of treatment have not been formally established
  - Addition of cyclosporine clearly improved outcomes compared with the use of ATG alone
  - Recently, certain other drugs like mycophenolate mofetil, sirolimus and cyclophosphamide are under trial.
- **For refractory AA:**
  - HSCT
  - Cyclophosphamide
  - Alemtuzumab-monoclonal anti-CD52 antibody (CD52 is an antigen present on all mature lymphocytes) and causes profound immunosuppression by lymphocytotoxicity.
- **For moderate pancytopenia:**
  - Clinically, the course of moderate aplastic anemia is variable: Some patients progress to severe disease, others remain stable and may not require intervention
  - ATG and cyclosporine are more effective in combination
  - Daclizumab-humanized monoclonal antibody to the interleukin-2 receptor
  - Androgens are given when there is residual hematopoietic function/ patients cannot afford immunosuppressive therapy.
  - They increase telomerase activity in human lymphocytes and CD34 cells, acting through the estradiol receptor.

Response Criteria

- **Complete response:**
  - Neutrophils >1.5 × 10^9/L
  - Platelets >100 × 10^9/L
  - Hemoglobin value normal for age and sex
- **Partial response (PR):** Not meeting criteria for CR, but
  - Improvement in blood counts (hemoglobin: >8 g/dL)
  - Absolute neutrophil count >0.5 × 10^9/L
  - Platelet: >20 × 10^9/L

POINTS TO REMEMBER

- Quantitative and practical measurements of oligoclonal T cell activity and of hematopoietic stem-cell number and function guide treatment decisions.
- The study of genetic risk factors, affecting hematopoietic-cell function and the immune response will clarify how agents in the environment initiate and perpetuate the marrow destruction of aplastic anemia.
- The treatment of severe aplastic anemia (allogeneic stem-cell transplantation or immunosuppression) has improved dramatically over the last 35 years, and long-term survival of more than 75% of patients can be anticipated with either therapy.
- For transplantation, the immediate challenge is the extension of stem-cell replacement to all patients, regardless of age, with a histocompatible sibling and who lack a family donor using alternative stem-cell sources
- The success of umbilical cord-blood transplantation may be enhanced by larger pools.
- For immunosuppression, many new drugs have yet to be tested in aplastic anemia
- Aplastic anemia management is a real challenge in a country like India due to longer duration from diagnosis to treatment and cost of therapy.

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27 Flow Cytometric Evaluation of Acute Leukemia

INTRODUCTION TO FLOW CYTOMETRY

- Flow cytometry is a laser-based technology.
- It involves the analysis of optical and fluorescence characteristics of single particle (e.g. cells, nuclei, chromosomes) during their passage within a narrow, precisely defined liquid stream.
- A flow cytometer has five components:
  1. Laser is a source of monochromatic light.
  2. Sheath fluid carries and aligns the cells so that they pass in a single line through laser beam.
  3. Optical system and filters that regulate the light signal.
  4. Photomultiplier detectors generate data and convert fluorescence signals from light to electrical.
  5. Computer for data analysis.
- The technology used to create a single line of cells in the sheath fluid is known as ***hydrodynamic focussing***.
- The LASER light falling on the cell gets scattered in two directions leading to
  - *Forward scatter:* Indicates the size of the cell that is larger the cell more the Forward scatter. Thus, monocytes have greater forward scatter and lymphocytes have the least forward scatter.
  - *Side scatter:* Indicates granularity of the cell that is more the number and size of granules in the cell greater the side scatter. Thus, granulocytes like neutrophils and eosinophils have greater side scatter.
- Initial flow cytometers allowed only 3–4 color antibody panels.
- However, currently, 8-color and 10-color flow cytometers are routinely used at many centers.
- These instruments allow analysis of 12–14 cellular parameters in a single tube.

Applications of Flow Cytometer

- Immunophenotyping: Leukemia, lymphomas, plasma cell disorders
- Platelet disorders, e.g. ITP
- Red cell disorders: PNH, HS, Fetomaternal hemorrhage
- Immunological disorders: Wiskott-Aldrich syndrome

- DNA analysis
- Leukocyte analysis, e.g. HIV

Acute Leukemia

- In acute leukemia the main objectives of flow cytometric evaluation are as follows:
  - Identification and quantification of cell populations within a sample
  - Differentiation of normal from abnormal cells
  - Differentiation of reactive from neoplastic cells
  - Identification of the differentiation or maturation stage of cell population
  - Quantification of tumor infiltration.
- To achieve the above-mentioned objectives, there are certain prerequisites. These are as follows:
  - Knowledge of physical characteristics/antigen expression on normal cells
  - Ability to distinguish between different patterns of expression of antigens
  - Ability to identify aberrant antigen expression
  - Identification of a robust leukemic associated immunophenotype (LAIP).
- Broadly in acute leukemia flow cytometry is applied in the following areas:
  - Diagnosis
  - Prognosis
  - Aberrant expression
  - Treatment
  - Minimal residual disease.

Diagnosis

- In diagnosis following are done:
  - Determination of myeloid/lymphoid lineage
  - Subtyping of AML
  - Immunological classification of ALL (B/T cell)
  - Distinguish ALL from lymphoma spillover
  - Diagnosis of biphenotypic leukemia
  - APML shows a teardrop pattern
  - B lymphoblasts show negative to moderate positivity
  - T lymphoblasts show the same positivity as normal lymphocytes.

Approach

- The diagnostic approach to acute leukemia starts by looking at side scatter in CD45.
- The pattern of side scatter gives a clue regarding the type of blast population as follows (Fig. 27.1)
  - Myeloblasts show a higher side scatter due to higher granularity.

List of Common Markers Used in Flow Cytometry

- Myeloid markers: cMPO, CD13, CD33, CD117, CD15
- Monocytic markers: CD64, CD14, CD11b, CD11c, lysozyme
- Megakaryocytic: CD41, CD61, CD36
- B lymphoid markers: CD19, CD10, CD20, CD22, cCD79a
- T lymphoid markers: CD3, CD5, CD7, CD1a, CD2, CD4, CD8

- NK cells: CD56
- Erythroid: CD71, CD235a, CD105

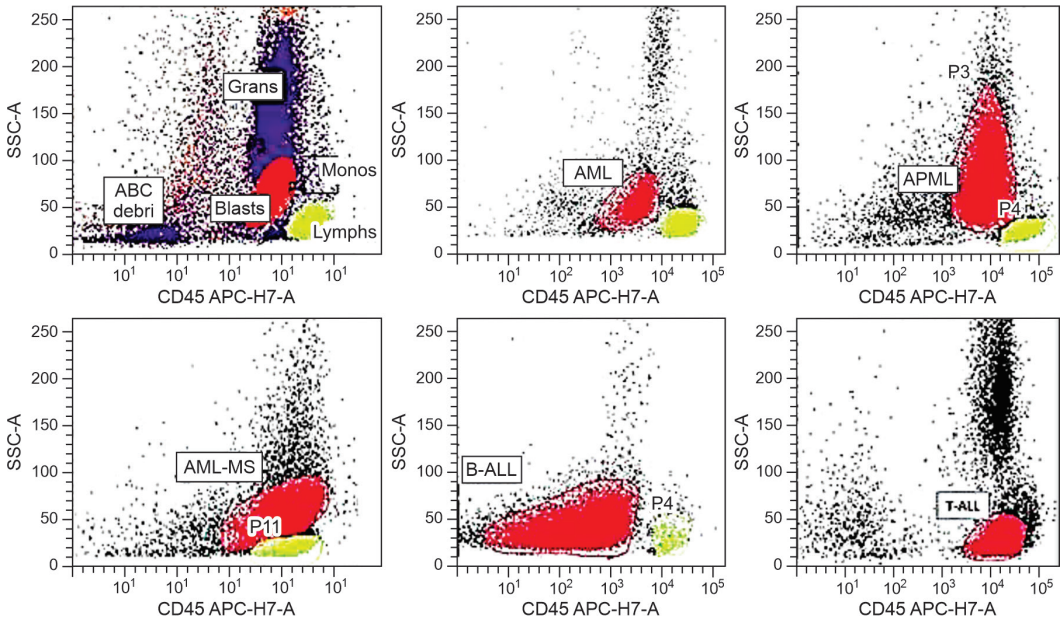

Fig. 27.1: CD45 expression of different types of blasts: myeloblasts—CD45 moderate with higher side scatter, APML teardrop pattern due to hypergranulations, AML M5 shows blasts in the myelomonocytic region, B lymphoblasts with heterogeneous dim CD45 and T lymphoblasts with CD45 expression in the same position as the normal lymphocyte population.

FLOW CYTOMETRY FINDINGS IN VARIOUS TYPES OF AML

AML with Recurrent Genetic Abnormalities

AML with t(8;21)(q22;q22.1) RUNX1-RUNX1T1

- Strongly express CD34, HLA-DR, MPO and CD13. However, there is a weak expression of CD33.
- They may also show expression of CD19 and PAX-5.

AML with inv(16)(p13.1q22) or t(16;16) (p13.1;q22);CBFB—MYH11

- There is presence of multiple blast populations.
- Blasts with high CD34 and KIT (CD117) expression and populations differentiating towards the granulocytic lineage (positive for CD13, CD33, CD15, CD65, and MPO).
- Another population that differentiates towards the monocytic lineage (positive for CD14, CD4, CD11b, CD11c, CD64, CD36, and lysozyme).

Acute Promyelocytic Leukemia with PML—RARA

- APL with PML—RARA (hypergranular variant) is characterized by low or absent expression of HLA—DR and CD34.
- It shows a homogeneous bright expression of CD33 and heterogeneous expression of CD13.

- Most cases show expression of KIT (CD117), although this is sometimes weak.
- The granulocytic differentiation markers CD15 and CD65 are negative or only weakly expressed and CD64 expression is common.
- In cases with microgranular morphology or the bcr3 transcript of the PML-RARA fusion gene, there is frequent expression of CD34 and CD2 by at least some cells.
- CD11c can also be expressed in some cells.
- CD2 expression in APL has been associated with FLT3–ITD.
- Expression of CD11b and CD11c can be upregulated after tretinoin treatment.

AML with t(9;11)(p21.3;q23.3);KMT2A–MLLT3

- In children they are associated with strong expression of CD33, CD65, CD4, and HLADR.
- Whereas expression of CD13, CD34, and CD14 is usually low.
- Most adult cases express some markers of monocytic differentiation, including CD14, CD4, CD11b, CD11c, CD64, CD36, and lysozyme.
- Whereas variable expression of markers of immaturity, such as CD34, KIT (CD117), and CD56 has been reported.

AML with t(6;9)(p23;q34.1); DEK—NUP214

- Blasts have a non-specific myeloid immunophenotype, with a consistent expression of MPO, CD9, CD13, CD33, CD38, CD123, and HLA-DR.
- Most cases also express KIT (CD117), CD34, and CD15.
- Some cases express the monocyte-associated marker CD64, and approximately half are TdT positive.

AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);GATA2, MECOM

- Blasts are positive for CD34, CD33, CD13, KIT (CD117), and HLA-DR.
- Most are CD38-positive, with aberrant CD7 expression frequently observed.
- High CD34 expression is more common with inv(3) than with t(3;3).
- A subset of cases may express megakaryocytic markers such as CD41 and CD61.

AML (megakaryoblastic) with t(1;22) (p13.3;q13.1); RBM15-MKL1

- The megakaryoblasts express one or more of the platelet glycoproteins: CD41 (glycoprotein llb/llla), CD61 (glycoprotein IIIa), and CD42b (glycoprotein lb).
- The myeloid associated markers CD13 and CD33 may also be positive.
- CD34, CD45, and HLA-DR are often negative.
- CD36 is characteristically positive but not specific.
- Blasts are negative with MPO antibodies.

AML with BCR-ABL1

- Demonstrates expression of myeloid antigens (CD13 and CD33) and CD34.
- Aberrant expression of CD19, CD7, and TdT is quite common.

AML with Mutated NPM1

- It is characterized by high CD33 expression and variable (often low) CD13 expression.
- KIT (CD117), CD123, and CD110 expression are common.

- HLA-DR is often negative.
- Two major subgroups of AML with mutated NPM1 have been described.
- One with an immature myeloid immunophenotypic profile and the other with a monocytic (CD36+, CD64+, CD14+) immunophenotypic profile.
- CD34 is negative in most cases but CD34+ cases do occur and have been associated with an adverse prognosis.
- A very small fraction of cells with the immunophenotype of leukemic stem cells (CD34+, CD38-, CD123+) is detectable in most patients.

AML with Biallelic Mutation of CEBPA

- HLA-DR, CD7, and CD15 show higher expression and there is a lower frequency of CD56 expression in cases with biallelic mutation as compared to cases with a single mutation.
- Monocytic markers such as CD14 and CD64 are usually absent.

AML with Mutated RUNX1

- Blasts usually express CD13, CD34, and HLADR.
- There is variable expression of CD33, monocytic markers, and MPO.

Acute Myeloid Leukemia with Myelodysplasia-Related Changes

- Results are variable due to the heterogeneity of the underlying genetic changes.
- Increase in CD14 expression on blast cells is related to a poor prognosis.
- An increased frequency of CD11b expression has been noted in patients with high-risk and monosomal karyotypes.
- Decreased expression of HLA-DR, KIT(CD117), FLT3 (CD135) and CD38 and increased expression of lactoferrin are associated with the presence of multilineage dysplasia.
- In cases with aberrations of chromosomes 5 and 7, a high incidence of CD34, TdT, and CD7 expression has been reported.
- In cases with antecedent MDS, CD34+ cells constitute only a subpopulation of blasts and may have a stem-cell immunophenotype, with low expression of CD38 and/or HLA-DR.
- An increase in the fraction of cells with this stem-cell immunophenotype has been correlated with high-risk cytogenetics and poor outcome.
- Blasts often express pan-myeloid markers (CD13, CD33), but aberrantly high or low expression of these markers is common.
- There is frequently aberrant expression of CD56 and/or CD7.

Therapy-related Myeloid Neoplasms (t-MN)

- There are no specific flow cytometry findings in t-MN.
- These reflect the heterogeneity of the underlying morphology.
- Blasts are generally CD34 positive and express pan-myeloid antigens such as CD13, CD33 and MPO.
- However, MPO expression has been reported to be downregulated in the neoplastic cells of some patients with t-MN.

- Immunohistochemical staining of p53-positive cells in bone marrow biopsies has been demonstrated to correlate well with TP53 mutations and with a poor prognosis {769,2983}.

AML NOS

Acute Myeloid Leukemia with Minimal Differentiation (FAB M0)

- Most cases express early hematopoietic associated antigens (e.g. CD34, CD38, and HLA-DR).
- They show a lack of antigens associated with myeloid and monocytic maturation, such as CD11b, CD15, CD14, and CD65.
- Blast cells express at least two myeloid-associated markers usually CD13 and KIT (CD117).
- Approximately 60% of cases express CD33.
- CD38 and/or HLA–DR expression may be decreased.
- There are no signs of monocytic differentiation, such as coexpression of CD64 and CD36.
- Blasts are negative for the B cell and T cell cytoplasmic lymphoid markers cCD3, cCD79a, and cCD22.
- MPO is negative by cytochemistry but may be positive in some blasts by flow cytometry or immunohistochemistry.
- Nuclear TdT is positive in approximately 50% of cases and has been suggested to be of favorable prognostic significance.
- CD7 expression is reported in approximately 40% of cases, but expression of other lymphoid-associated membrane markers is rare.

Acute Myeloid Leukemia without Maturation (FAB M1)

- Blasts express MPO and one or more myeloid-associated antigens, such as CD13, CD33, and KIT (CD117).
- CD34 and HLA-DR are positive in approximately 70% of cases.
- Usually no expression of markers associated with granulocytic maturation (e.g. CD15 and CD65) or monocytic markers (e.g. CD14 and CD64) is seen.
- CD11b is expressed in some cases.
- Blasts are negative for the B cell and T cell cytoplasmic lymphoid markers cCD3, cCD79a, and cCD22.
- CD7 is found in about 30% of cases, and expression of other lymphoid-associated membrane markers (e.g. CD2, CD4, CD19, and CD56) has been described in 10–20% of cases.

Acute Myeloid Leukemia with Maturation (FAB M2)

- Leukemic blasts usually express one or more of the myeloid-associated antigens CD13, CD33, CD65, CD11b, and CD15.
- Flow cytometric analysis reveals features of granulocytic differentiation.
- There is often expression of HLA-DR, CD34, and/ or KIT (CD117), which may be present only in some blasts.
- Monocytic markers such as CD14, CD36, and CD64 are usually absent.
- CD7 is expressed in 20–30% of cases.

- CD56, CD2, CD19, and CD4 are uncommon expressed (seen in ~10% of cases) and may be found only in the most immature blasts.

Acute Myelomonocytic Leukemia (FAB M4)

- It shows several populations of blasts variably expressing the myeloid antigens CD13, CD33, CD65, and CD15.
- One of the blast populations is usually also positive for some markers, characteristic of monocytic differentiation, such as CD14, CD64, CD11b, CD11c, CD4, CD36, CD68, CD163, and lysozyme.
- Co-expression of CD15, CD36, and strong CD64 is characteristic of monocytic differentiation.
- A population of immature blasts that express CD34 and/or KIT (CD117) is observed.
- Most cases are positive for HLA-DR and approximately 30% for CD7.
- Expression of other lymphoid-associated markers is rare.

Acute Monoblastic and Monocytic Leukemia (FAB M5)

- There is variable expression of myeloid antigens CD13, CD33 (often very bright), CD15, and CD65.
- There is generally an expression of at least two markers characteristic of monocytic differentiation, such as CD14, CD4, CD11b, CD11c, CD64 (bright), CD68, CD36 (bright), and lysozyme.
- CD34 is positive in only 30% of cases, whereas KIT (CD117) is more often expressed.
- Most cases are positive for HLA–DR.
- MPO can be expressed in acute monocytic leukemia and less often in monoblastic leukemia.
- Aberrant expression of CD7 and/or CD56 is found in 25–40% of cases.

Pure Erythroid Leukemia (FAB M6)

- The erythroblasts usually express glycophorin and haemoglobin A, as well as the less lineage-specific marker CD71.
- E-cadherin, which stains early erythroid forms, is positive in the vast majority of cases and is specific for erythroid differentiation.
- The blasts are often positive for KIT (CD117) and usually negative for HLA-DR and CD34.
- CD36 is positive in most cases, but is not specific for erythroblasts and can be expressed by monocytes and megakaryocytes.
- Antigens associated with megakaryocytes (i.e. CD41 and CD61) are typically negative.

Acute Megakaryoblastic Leukemia (FAB M7)

- The megakaryoblasts express one or more of the platelet glycoproteins: CD41 (glycoprotein llb/llla), CD61 (glycoprotein Ilia), and CD42b (glycoprotein lb).
- The myeloid-associated markers CD13 and CD33 may be positive.
- CD34, CD45, and HLA-DR are often negative, especially in children.
- CD36 is characteristically positive but not specific.
- Blasts are negative with MPO antibody and with other markers of granulocytic differentiation.

- Lymphoid markers and TdT are not expressed, but there may be an aberrant expression of CD7.
- Cytoplasmic expression of CD41 or CD61 is more specific and sensitive than is surface staining.

Acute Basophilic Leukemia

- Blasts express myeloid markers such as CD13 and CD33.
- They are usually positive for CD123, CD203c, and CD11b; and are negative for other monocytic markers and KIT (CD117).
- Blasts may express CD34.
- They may be positive for HLA-DR but are negative for KIT which is contrary to what is seen in normal basophils.
- Blasts usually express CD9.
- Some cases are positive for membrane CD22 and/or TdT.
- Other membrane and cytoplasmic lymphoid-associated markers are usually negative.

Acute Panmyelosis with Myelofibrosis

- Flow cytometric evaluation depends on the adequacy of the bone marrow specimen obtained or if circulating blasts are present.
- The cells show phenotypic heterogeneity, with varying degrees of expression of myeloid-associated antigens.
- Blasts usually express the progenitor/early precursor marker CD34 and one or more myeloid associated antigens: CD13, CD33, and KIT (CD117).
- MPO is usually negative in the blasts.
- In some cases, some of the immature cells express erythroid antigens.
- Immunohistochemistry is often used in these cases.

FLOW CYTOMETRY FINDINGS IN VARIOUS TYPES OF ALL

B-ALL

- The flow cytometry panel used to further categorize B-ALL includes the following antibodies:
  - HLA-DR
  - TdT
  - CD10
  - CD19
  - SmIg
  - CyCD79a
- The results of this panel and the final diagnosis rendered is shown in Table 27.1.

Table 27.1: Flow cytometric diagnosis of B-ALL

Subtype	HLA-DR	TdT	CD10	CD19	SmIg	Cy CD79a
Pro-B-ALL	+	+	–	+	–	+
Common ALL	+	+	+	+	–	+
Pre-B-ALL	+	–	+	+	CmIg	+
Mature B-ALL	+	–	+/–	+	Kappa/Lambda	+

- t(12;21) correlates on flow cytometry with CD10 positive, low expression of CD19, CD20 negative, and aberrant expression of CD13.
- Hyperdiploidy, on the other hand, is associated with CD19, CD20 positive and CD45 negative.
- Both of these show favorable prognosis.
- t (9;22) shows an aberrant expression of myeloid markers CD13, CD33 and CD45 negative.
- MLL translocation t (4;11) shows a lack of CD10, CD20, frequent aberrant expression of CD15.
- t (1;19) (q23;p13) demonstrates lack of CD34.
- All these above three are associated with unfavorable prognosis.
- CD20 expression in adult pre-B-ALL is associated with a poor prognosis.

Hematogones

- These are physiological precursors of maturing B cells.
- They consist of <1% of bone marrow cells.
- Morphologically resemble lymphoblasts.
- The conditions in which they are seen include the following:
  - Autoimmune/congenital cytopenias
  - Solid organ tumors, e.g. neuroblastomas
  - AIDS
  - NHL
  - Post-chemotherapy
  - Post-bone marrow transplantation
  - Copper deficiency.
- They show variable expression of the following antigens:
  - CD10, CD 20, CD34, CD19, dimly positive for CD22
- J-shaped trail pattern on flow cytometry is seen with CD10/CD20.
- There is the presence of heterogeneous, varying intensity from negative to bright positivity.
- TdT and CD34 show variable positivity in hematogones.
- TdT shows uniform positivity and CD19 shows variable positivity in ALL.

T-ALL

- As far as the T-ALL is concerned the important markers which are expressed are TdT, CD1a, CD2, cCD3, CD4, CD5, CD7, CD8.
- Out of these cCD3 is highly specific.
- CD4, CD8 co-expression is also observed.
- The precursor markers that may be positive are TdT, CD99, CD34 and CD1a.
- Important differential diagnosis is NK precursor ALL which is CD56 positive.
- The diagnosis of precursor NK-ALL/LBL may be considered in a case that shows the following:
  - Expression of CD56 along with
  - Immature T-associated markers CD7 and CD2, even cCD3.
- However, it is extremely important that there should be the absence of B cell and myeloid markers and blastic plasmacytoid dendritic cell neoplasm is ruled out.
- Bright CD45 expression is associated with a bad prognosis with high chance of relapse in cases of both B and T cell ALL.

- It may show aberrant expression of CD13, CD33 and CD79a.
- T-ALL with activating mutations of FLT 3 is positive for CD117.

Minimal Residual Disease

- Minimal residual disease (MRD) refers to the small number of malignant cells that remain after therapy when the patient is in complete remission and shows no symptoms or overt signs of disease.
- The tests that are available to detect MRD with their sensitivities are shown in Table 27.2.

Table 27.2: MRD detection methods and their sensitivities

Test	*Sensitivity*
Karyotyping	10^{-2}
FISH	10^{-3}
Flow cytometry	10^{-4}
Reverse transcription PCR	10^{-5} More sensitive and specific
Real-time quantitative PCR	10^{-6} Most sensitive and specific
NGS	10^{-6} Most sensitive and specific

- Currently flow cytometry is used in two different approaches for MRD detection:
  - Establishing the presence of **leukemia-associated aberrant immunophenotype (LAIP)** at diagnosis or relapse and use this information at specified time points for detection of MRD traces.
  - Apply a standardized panel of antibody combinations for all MRD cases, in a different-from-normal approach.
- In the LAIP approach, the log ratio of LAIP-positive cells at follow-up are compared to the initial proportion of cells carrying the respective LAIP.
- Hence, LAIP suitable for disease monitoring must be established at the time of diagnosis using comprehensive antibody panels for all cases.
- The same information must be used at the time of MRD estimation.
- In the different-from-normal approach, a standardized panel of antibody combinations and using healthy control bone marrow samples, the patterns of expression of antigens are delineated and templates are made.
- The biaxial dot plots of chosen combinations of markers will contain consistently blank areas when normal bone marrow cells are analyzed.
- MRD will be indicated by positive readings in these areas.
- Normal marrow cells and leukemic blast cells showing similar immunophenotypes can be distinguished using their quantitative differences.
- For example, the overexpression of CD10 and CD34, underexpression of CD45 and CD38 in CD19 positive blasts are abnormal features in some B-lineage ALL cases.

Flow Cytometry in MRD-All

- The immunophenotypic markers for MRD detection in childhood ALL have been standardized as shown in Table 27.3.

- However, as far as the adult ALL is concerned still more work is required to standardize the MRD detection panel.
- The normal equivalents of T lineage ALL cells are immature T cells which are confined to the thymus.

Table 27.3: Antibodies used for MRD detection in ALL

ALL lineage	*Type of phenotypic abnormality*	*Markers*	*Frequency (%)*
B	Overexpression or under-expression of markers also expressed in normal B cell progenitors	CD19/CD34/CD10/TdT CD19/CD34/CD10/CD22 CD19/CD34/CD10/CD38 CD19/CD34/CD10/CD45 CD19/CD34/CD10/CD58	30–50 20–30 30–50 30–50 40–60
	Expression of markers not expressed in normal B cell progenitors (aberrant marker)	CD19/CD34/CD10/CD13 CD19/CD34/CD10/CD15 CD19/CD34/CD10/CD33 CD19/CD34/CD10/CD65 CD19/CD34/CD10/CD56 CD19/CD34/CD10/CD66c CD19/CD34/CD10/7.1	10–20 5–10 5–10 5–10 5–10 10–20 3–5
	Expression of markers expressed at different stages of normal B cell maturation	CD19/CD34/CD10/CD21 CD19/CD34/TdT/cyto-plasmic heavy chain μ	5–10 10–20
T	Phenotypes normally confined to the thymus	TdT/CD3 CD34/CD3	90–95 30–50

- Thus, the aim of MRD studies in T-ALL is to search for these cells in the bone marrow or peripheral blood.
- Combination of TdT with cytoplasmic or surface CD3/CD5/CD7.
- In case of weak or negative TdT expression CD34 is used.
- CD19 and HLA DR are usually not expressed on T-ALL blasts but are strongly positive on most normal bone marrow TdT+ cells.
- This helps in differentiating normal from leukemic cells.
- CD99 overexpression is either non-existent or extremely rare on T cells elsewhere than thymus.
- It is claimed to be a highly informative marker for MRD.
- There is an added advantage of surface expression in contrast to Tdt.
- Thus, the MRD detection panel may consist of CD99/CD5/CD3/sCD3.
- In B-precursor ALL with respect to immunophenotyping the most frequently expressed aberrant phenotypes involve TdT dim/CD10+ and CD38 dim/CD34+.
- CD19 is a useful additional gating marker.
- In resource-limited settings a three-color combination of CD19/CD10/CD34 is recommended on day 19 of induction.
- It is highly significant for predicting relapse.
- Normal CD19 cells become undetectable after 2 weeks (<0.01%) of chemotherapy in B-ALL patients.
- Thus, if present they indicate the presence of residual cells.
- LFA3 (lymphocyte function-associated antigen-3) is found to be overexpressed in childhood B-ALL.
- It may prove to be an attractive tool for MRD.

Detection of Residual Disease in CSF

- Approximately **5% to 10%** of patients with ALL who enter remission will have an isolated CNS relapse.
- Some of these patients may also have residual leukemia in the BM.
- The **first sign of CNS involvement** is often the presence of **malignant cells** in the CSF.
- It is very difficult to discriminate between leukemic lymphoblasts and normal activated lymphocytes by routine morphologic examination.
- Although B, T-lymphocytes and monocytes are normally found in the CSF, cells expressing TdT should indicate CNS involvement.

Flow Cytometry in MRD-AML

- AML cells usually spread across many areas of the dot plot instead of forming a tight cluster.
- This is in contrast to acute lymphoblastic leukemia (ALL), which is usually more homogeneous.
- The most relevant types of aberrations in AML for MRD detection are as follows:
  - **Asynchronous antigen expression:** It is simultaneous expression of early and late markers such as co-expression of CD34 and CD15 antigens.
  - **Lineage infidelity:** This means expression of lymphoid-associated markers, i.e. CD2, CD3, CD5, CD7, CD10, and CD19 on myeloid blast cells.
  - Antigen overexpression: It indicates abnormally increased expression of a certain antigen per cell.
  - **Aberrant light-scatter properties:** This is an expression of lymphoid-associated antigens in blast cells displaying a relatively high forward scatter and side scatter, corresponding to normal myeloid cells.
  - **Absence of lineage-specific antigens:** Absence of antigen expression such as CD13 and CD33 on myeloid blasts.
- The antibody combination used for MRD studies by flow cytometer in AML along with their sensitivity is shown in Table 27.4.

Table 27.4: Antibody panels used for MRD detection in AML

LAIP class	*Examples*	*% of expression*
Cross lineage expression (typically of lymphoid antigen)	CD33+, CD2+, CD34+, CD34+, CD13+, CD19+	34%
Antigen overexpression	HLA-DR, CD33, CD34 CD64, CD4, CD45	9%
Loss of antigen expression	HLA-DR-, CD33+, CD34+	30%
Asynchronous antigen expression	CD15+, CD33+, CD34+ CD65+, CD33+, CD34+	83%

- Blasts should be identified by a CD45/side scatter gating strategy.
- When possible, an extensive panel of different myeloid and lymphoid markers should be used in combination to increase the sensitivity of LAIP detection.
- In theory 1 leukemic cell in 10^6 nucleated cells can be identified.

- However, a more realistic figure for practical applications is 1 in 10^4.
- The level of sensitivity varies (from 10^{-3} to 10^{-5}) depending on the type of phenotypes being analyzed and the combination of antibody reagents used for their detection.

POINTS TO REMEMBER

- There is an increasing importance of molecular and genetic features in the sub-classification of acute leukemias.
- However, morphologic and immunophenotypic analysis remains the main modality to diagnose acute leukemia.
- In the initial evaluation it provides a rapid assessment to direct specific molecular genetic tests.
- Flow cytometric immunophenotyping may directly correlate with prognosis and in an era of novel agents may help in the development of monoclonal antibodies to the tumor antigens.
- It is the mainstay of evaluating minimal residual disease, particularly in cases without any specific molecular signature.
- Flow cytometry provides a direct assessment of the varied surface antigen expression on hematolymphoid neoplasms.
- Thereby, facilitating a rational and individualized selection of such targeted immunotherapy strategies.

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Inherited Platelet Disorders

28

INTRODUCTION

- Osler, Hayam, and Bizzozero first identified platelets.
- James Homer Wright described them as hematopoietic components arising from megakaryocytes.
- William Duke in 1910 described 3 patients with low platelet counts that had hemorrhagic disease.
- Venous shunt was made from a normal donor to a thrombocytopenic recipient and showed that the platelet count could rise and the bleeding would cease.
- Size of platelets varies from 2–4 microns.
- They are small, granulated, non-nucleated and round to oval in shape.
- Normal count is 1,50,000 – 4,50,000/mm^3.
- Lifespan averages 8 days.

FUNCTIONAL ORGANIZATION

- Platelets are divided into four structural regions
  1. Peripheral zone
  2. Sol-gel zone
  3. Organelle zone
  4. Membrane system.

Peripheral Zone

- It consists of an external (exterior) coat, unit membrane and sub-membranous area.
- It maintains platelet integrity, receives and transmits stimuli triggering platelet responses (adhesions, aggregations).
- Cell membrane is 6 nm thick.
- It is composed of carbohydrates (glycocalyx), proteins (glycoproteins) and lipids (phospholipids, cholesterol and glycolipids)
- Out of all of these glycoproteins and phospholipids are functionally most important.
- It contains mainly two kinds of substances:
  - Glycoprotein receptors for collagen, fibrinogen and vessel von-Willebrand factor.
  - Phospholipids include platelet factor 3.

- Glycoproteins have the following functions:
  - Prevent the adherence of platelets to normal endothelium.
  - Accelerate the adherence of platelets to collagen and damaged endothelium in ruptured blood vessels.
  - Form a receptor for ADP and thrombin.
- Numerous G-protein receptors or adhesion receptors (integrins) are present on the cell surface.
- These are transmembrane heterodimers composed of alpha and beta subunits, responsible for adhesion and signal transduction.
- Glycoproteins are designated I (large) to IX (small).
- The alphabets a and b were added when electrophoretic techniques allowed for the resolution of single bands to separate bands.
- Glycoprotein receptors:
  - *GP Ib-V-IX:* It is a complex of four gene products, that serves as a receptor for vWF thereby mediating platelet adhesion. Defect/deficiency leads to Bernard-Soulier syndrome.
  - *GP IIb-IIIa:* It is the most abundant and recognizes four different molecules: fibrinogen, fibronectin, vitronectin, and vWF. It is involved in platelet aggregation. Defect/deficiency causes Glanzmann's thrombasthenia.
  - *Others:* GP Ia, IIa and GP VI. These act as collagen receptors.

Sol-gel Zone

- It is a matrix of platelet cytoplasm which contains microfilaments and microtubules.
- These function as cytoskeletal elements.
- Microfilaments and microtubules maintain the discoid platelet shape.
- Contractile system for shape change, pseudopod formation, internal contractions and granule secretion is also due to these elements.

Microtubules

- They are made up of tubulins (proteins).
- They are responsible for the discoid shape of the platelets.

Organelle Zone

- This zone is composed of all internal platelet components, except microtubules, microfilament components and membrane system.
- The major components are platelet granules.
- These are morphologically and biochemically heterogeneous.
- Under light microscopy they are azurophilic granules (alpha-granules under electron microscope).
- Other components include:
  - Contractile proteins
  - Golgi apparatus
  - Endoplasmic reticulum
  - Mitochondria
  - Lysosomes
  - Glycogen granules
  - Enzymes
  - Chemical substances

- The major contractile proteins are as follows:
  - Actin
  - Thrombosthenin
  - Myosin.
- They enable activated platelets to change their shape.
- The residuals of endoplasmic reticulum and Golgi apparatus function in the synthesis of enzymes and calcium storage.
- Mitochondria and enzyme system carry out the synthesis of ATP and ADP.
- Lysosomes contain hydrolytic enzymes.
- Glycogen granules are used for the anaerobic production of energy.
- Enzyme system synthesizes prostaglandins from phospholipids of the platelet membrane.

Membrane System

- It has two parts:
  - Open canalicular system
  - Dense tubular system
- The open canalicular system is responsible for the passage for externalization of platelet secretory products and internalization of substances from plasma into the platelet.
- The dense tubular system carries out sequestration of Ca^{2+}, localization of enzymes for prostaglandin synthesis.
- These trigger platelet aggregation.
- Important chemical substances present in the cell membrane are as follows:
  - Calcium ions
  - Magnesium ions.
  - Adenosine triphosphate (ATP)
  - Adenosine diphosphate (ADP)
- Types of platelet granules:
  - *Dense granules:* They contain non-protein substances like ATP, ADP, Ca++ and serotonin.
  - *Alpha granules:* These contain the secreted proteins for example clotting factors, fibrin stabilizing factor XIII and platelet-derived growth factor.
- The various platelet constituents and their functions are shown in Table 28.1.

Table 28.1: Locations and functions of various platelet constituents

Platelet location	*Constituent*	*Function*
Exterior coat	Fibrinogen	Platelet aggregation
Membrane	Arachidonic acid	Prostaglandin synthesis
	Platelet factor 3 (phospholipid)	Enhances coagulation
	cAMP	Inhibits release reaction
Microtubules	Tubulin	Cytoskeleton contractility
Microfilaments	Thrombosthenin	Shape change, clot reaction
Alpha-granules	Beta-thromboglobulin	Impedes prostacyclin production endothelial cells
	Catalase	Enzymatic process

Contd.

Table 28.1: Locations and functions of various platelet constituents		
Platelet location	*Constituent*	*Function*
	Factor VIII-related antigen	Platelet adhesion to the subendothelium
	Fibrinogen	Shape change, clot reaction, release reaction
	Fibronectin	Adherence to extracellular matrix Promote wound healing

PLATELET FUNCTIONS

- The major role of platelets is in the process of primary hemostasis.
- It is an extremely dynamic, complicated, and continuous interaction between vessels, platelets, and plasma components.
- The process includes the following three steps:
  1. Adhesion
  2. Activation (secretion)
  3. Aggregation

Adhesion

- Vascular injury exposes the pro-coagulant components of the sub-endothelial extracellular matrix chiefly collagen, proteoglycans, and fibronectin.
- Platelets are exposed to these components in a rolling fashion.
- vWF acts as an adhesion bridge between the platelet GPIb-V-IX complex and exposed collagen.
- Platelets also adhere to fibronectin.
- However, vWF-GPIb bridge is the only association strong enough to overcome blood flow shearing force.

Secretion

- The change in the shape of platelet occurs due to formation of pseudopods.
- Platelet granule contents are released through the canalicular system.
- Cytoplasmic activation of the eicosanoid pathway leads to increased synthesis of thromboxane A_2, decreased cAMP, and mobilization of Ca^{++}.
- Phospholipids act as the binding surface for factors Va and Xa (along with Ca^{++}). Thus, forming the prothrombinase complex.
- This is an extremely crucial step in secondary hemostasis.

Aggregation

- It is promoted by ADP and TXA_2 release.
- ADP induces a conformational change of the GP IIbIIIa receptor, allowing fibrinogen binding.
- Platelets aggregate via fibrinogen bound to GP IIbIIIa receptors.
- This is an auto-catalytic reaction, thereby activating other platelets.
- Formation of a primary hemostatic plug.
- The process of formation of a stable hemostatic plug is shown in Fig. 28.1.

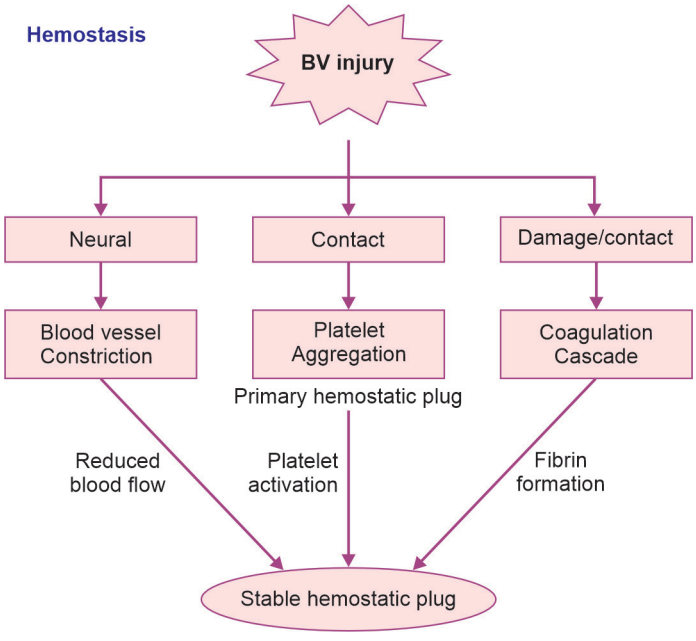

Fig. 28.1: Formation of a stable hemostatic plug by primary hemostasis

THROMBOPOIESIS

- It is the process of formation of platelets.
- Thrombopoietin derived from the kidney or liver acts on the myeloid stem cell which differentiates into a megakaryoblast.
- It has a large, oval, kidney-shaped nucleus which is 20–30 microns in diameter.
- Megakaryoblast further differentiates into a megakaryocyte.
- It has a multilobed (4–16 lobes) nucleus.
- This occurs due to the process of endomitosis in which mitosis occurs due to separation of nuclei.
- The cell margin shows pseudopodia.
- Platelets are derived from the pseudopodia of the megakaryocyte.
- Each megakaryocyte forms up to 4000 platelets.
- The whole process of formation of platelets from stem cells takes 10 days.

Thrombopoietin (TPO)

- It is the primary regulatory protein in the production of platelets.
- TPO has a major effect on almost all steps of megakaryocyte differentiation and maturation as mentioned below:
  - Promotion of growth of megakaryocytic colony-forming cells (Meg-CFC).
  - Increased rate of endomitosis.
  - Stimulation of megakaryocyte maturation.
- TPO gene is located on chromosome 3.
- It is derived from liver, kidneys and smooth muscle cells.
- Half-life is 30 hours.

- In circulation, most of it gets removed by thrombopoietin (c-Mpl) receptors on platelets and possibly some by bone marrow megakaryocytes.
- The residual thrombopoietin (50 to 150 pg/mL) provides basal stimulation of megakaryocytes and a basal rate of platelet production.

TYPES OF BLEEDING DISORDERS

- These include the following:
  - Disorder of primary platelet plug formation
  - Fibrin formation
  - Premature clot dissolution—fibrinolysis
- Spontaneous skin petechiae are usually seen in severe thrombocytopenia.
- Spontaneous hemarthrosis usually indicates coagulation factor deficiency.

INHERITED PLATELET DISORDERS

- They can be broadly classified into four types as shown in Table 28.2.

Table 28.2: Classification of inherited platelet disorders

Type of defect	*Name of disease(s)*
Adhesion defect	Bernard-Soulier syndrome von Willebrand disease
Aggregation defect	Glanzmann's thrombasthenia Afibrinogenemia
Secretion disorder	**Alpha granule**: Gray platelet syndrome **Delta granule**: Storage pool defects Chédiak-Higashi disorder, Wiskott-Aldrich syndrome, thrombocytopenia with absent radius (TAR) syndrome
Disorder of procoagulant activities	Scott syndrome

Bernard-Soulier Syndrome

- First described in 1948 by Jean Bernard and Jean-Pierre Soulier who were French hematologists.
- It is an autosomal recessive condition characterized by moderate to severe thrombocytopenia, giant platelets, and perfuse/spontaneous bleeding.
- Basis for the disease is deficiency or dysfunction of the GPIb-V-IX complex.
- Decreased GPIb-V-IX leads to decreased platelet adhesion to the subendothelium via decreased binding of vWF.
- Approximately 20,000 copies of GPIb-V-IX are present per platelet.
- GPIb is a heterodimer with an alpha and beta subunit.
- The gene for GPIb-alpha is located on chromosome 17, GPIb beta on chromosome 22 and GPIX and V on chromosome 3.
- Most mutations are missense or frameshifts resulting in premature stop codons.
- Most mutations involve GPIb expression (rare GPIX mutations have been described; no mutations in GPV reported to date).

Lab Diagnosis

The findings in lab investigations include:

- Prolonged bleeding time
- Thrombocytopenia (Plt <20,000)
- Peripheral smear shows large platelets (mean diameter >3.5 microns).

von Willebrand Disease

- This disease is named after Dr Erik von Willebrand who first described this hereditary bleeding disorder in 1924.
- He distinguished it from hemophilia.
- The von Willebrand factor protein was purified in the 1970s.
- It is produced in megakaryocyte, endothelium and subendothelial connective tissue.
- The size of the protein varies from 250 kDa for monomer to more than 20,000 kDa for multimer.
- It has functions of binding for platelet, FVIII, and collagen.
- vWF binds platelets to exposed collagen.
- It acts as a chaperone of FVIII.
- It is cleaved by ADAMTS-13 protein at A2 domain and eliminated by liver and spleen.
- The clinical manifestations are due to platelet binding problems or severely decreased FVIII half-life.
- It presents with mucocutaneous bleeding.
- Clinical presentation is like hemophilia in type 2N and type 3.
- There is a history of bleeding diathesis in first-degree relatives.
- Significant bleeding may be determined by bleeding score of 3 in male and 5 in female.
- The mode of inheritance is autosomal dominant.
- Exceptions are type 2N and type 3 which are inherited in autosomal recessive mode.

Lab Diagnosis

- Platelet aggregation studies show normal aggregation in response to all agonists except Ristocetin.
- However, it should be kept in mind that Pseudo vWD also shows similar results and can only be diagnosed by molecular investigations.
- Flow cytometry shows decreased expression of mAbs to CD42b (GPIb), CD42a (GPIX) and CD42d (GPV).
- The goal is to prevent bleeding.

Management

- Patients with vWF level > 40 UI/dL or without a history of bleeding are not at risk of bleeding.
- Desmopressin is the first line of treatment of vWD.
- Desmopressin infusion is indicated if vWF: Rco/Ag ratio is near normal and vWF level > 30 UI/dL.
- For patients with type 2B or type 3 and patients who do not respond to desmopressin factor replacement is required.

Glanzmann's Thrombasthenia

- It was first described by Eduard Glanzmann (1887–1959) who was a Swiss pediatrician.
- The disease is inherited in an autosomal recessive manner.
- The genes of both of these proteins are on chromosome 17.
- Different genetic mutations of either GP IIb or IIIa genes result in a heterogeneity of thrombasthenia phenotype.
- Carrier detection in GT is important to control the disease in family members.
- It can also be acquired as an autoimmune disorder.

Pathogenesis

- Platelet glycoprotein IIb/IIIa (GP IIb/IIIa) complex is either deficient or present but dysfunctional.
- Defect in the GP IIb/IIIa complex leads to defective platelet aggregation and subsequent bleeding.
- Aggregation of platelets occurs in response to ristocetin, but not to other agonists such as ADP, thrombin, collagen, or epinephrine.
- Glanzmann thrombasthenia has three categories of severity, depending on the extent of the platelet deficiency in Glycoprotein IIb/IIIa.
  1. Type I (Severe): A level less than 5% of normal
  2. Type II (Less severe): A level between 5% and 20% of normal
  3. Type III (Least severe): A variant of thrombasthenia with levels of more than 50% of normal, but with major abnormalities in the way platelets aggregate.
- Clinical symptoms include:
  - Purpura
  - Menorrhagia
  - Mucosal bleeding
  - Brain hemorrhages
  - Epistaxis
  - Gingival bleeding
  - Gastrointestinal bleeding
  - Postpartum bleeding
  - Increased bleeding postoperatively.

Lab Diagnosis

- CBC shows a normal platelet count and morphology.
- However, bleeding time is greatly prolonged.
- ***Platelet aggregation test***: There is an absence of platelet aggregation in response to ADP, collagen, epinephrine, or thrombin.
- Flow cytometry shows no expression of CD 41 and CD 61 but CD 42b shows normal expression.
- Studies of GP IIb/IIIa receptors on the PLT membrane can also be performed.

Management

- Dental hygiene lessens gingival bleeding.
- Avoidance of antiplatelet agents such as aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and naproxen, and anticoagulants.
- Iron or folate supplementation.

- Antifibrinolytic drugs such as tranexamic acid or ε-aminocaproic acid.
- Desmopressin (DDAVP) does not normalize the bleeding time in Glanzmann's thrombasthenia but anecdotally improves hemostasis.
- Hormonal contraceptives to control excessive menstrual bleeding.
- Platelet transfusions are to be given only when bleeding is severe since there is a risk of platelet alloimmunization.
- Recombinant factor VIIa.
- Hematopoietic stem cell transplantation (HSCT) for severe recurrent hemorrhages.

STORAGE POOL DEFECTS

- They are classified by the type of granular deficiency or secretion defect.
- The more common types include Dense body deficiency, alpha granule deficiency (gray platelet syndrome), mixed deficiency and Factor V Quebec.

Dense Body Deficiency

- It is characterized by a decreased number of dense bodies that contain ADP, ATP, calcium, pyrophosphate and serotonin.
- Normal platelet contains 3–6, 300-micron size dense bodies.
- This is described in inherited disorders like Hermansky-Pudlak syndrome, Wiskott-Aldrich syndrome, Chédiak-Higashi syndrome, and thrombocytopenia with absent radius (TAR) syndrome

Wiskott-Aldrich Syndrome

- It is an X-linked, genetic defect in the Wiskott-Aldrich syndrome protein (WASP).
- This protein is responsible for actin cytoskeleton formation in hematopoietic cells.
- It is characterized by thrombocytopenia (with platelet storage pool defect), eczema and recurrent infections.

Hermansky-Pudlak Syndrome

- It was described in 1959 by Hermansky and Pudlak.
- Mode of inheritance is autosomal recessive.
- It is characterized by tyrosinase-positive oculocutaneous albinism, ceroid-like deposition in lysosomes of the reticuloendothelial system and marrow.
- Highest prevalence reported in Puerto Rico.
- It may be associated with pulmonary fibrosis, inflammatory bowel disease and recurrent infections.
- A quantitative deficiency of dense granules leads to mild to moderate bleeding diathesis.

Chédiak-Higashi Syndrome

- It was described by Beguez Cesar in 1943, Steinbrinck in 1948, Chédiak in 1952, and Higashi in 1954.
- Mode of inheritance is autosomal recessive.
- There is abnormal microtubule formation and giant lysosomal granules are present in phagocytes and melanocytes.

- No degranulation/chemotaxis leads to recurrent bacterial infections.
- Partial oculocutaneous albinism is also seen.
- Dense-body granules are decreased/absent.

Thrombocytopenia with Absent Radius (TAR)

- First described in 1951
- Mode of inheritance is autosomal recessive.
- It is characterized by absent radii, thrombocytopenia (with storage pool defect), along with other abnormalities of the skeletal, GI and cardiovascular systems.
- Etiology is unclear.
- Hemorrhage is the major cause of mortality.
- Prognosis is good if the child survives the first two years.

Lab Diagnosis

- Platelet aggregation studies may show diminished response to low-concentration collagen.
- ADP and epinephrine show diminished second-wave response.
- Ristocetin shows normal aggregation.
- EM shows lack of dense bodies.
- Increased ATP:ADP ratio within platelets.

ALPHA GRANULE DEFICIENCY

- The other names include Alpha storage pool deficiency and Gray platelet syndrome.
- It was first described by Raccuglia in 1971.
- Normal platelets contain approximately 50 alpha granules.
- These granules contain PF4, beta-thromboglobulin, PDGF, fibrinogen, vWF, factor V and fibronectin.
- Patients lack granules and present with lifelong, mild to moderate mucocutaneous bleeding.

Lab Diagnosis

- Prolonged bleeding time with mild thrombocytopenia.
- Agranular, large "gray" platelets on peripheral smear.
- Aggregation studies show decreased to absent response to collagen.

MISCELLANEOUS CONDITIONS

Fechtner Syndrome

- It is characterized by macrothrombocytopenia and neutrophilic inclusions.
- Additional features include nephritis, sensorineural hearing loss and cataracts.

Epstein Syndrome

- It is characterized by macrothrombocytopenia but absent neutrophilic inclusions.
- Additional features include nephritis and sensorineural hearing loss but no cataracts.

MYH9-related Disorders

- These disorders present with only mildly increased bleeding.
- They are usually diagnosed during routine testing of an asymptomatic individual.
- Mode of inheritance is autosomal dominant.
- Does not usually require any therapy.
- Educating the affected families about this diagnosis is paramount to avoid potentially dangerous treatments for presumed ITP.

CATCH22

- These patients show mild macrothrombocytopenia.
- No serious bleeding is observed.
- It is characterized by cardiac abnormalities, parathyroid and thymus insufficiencies, cognitive impairment and facial dysmorphology.
- The basic molecular defect is deletion of GP1BB gene located on chromosome 22q11.
- There is no need of therapy for these mild thrombocytopenias.
- However, it is important to recognize these conditions to avoid unnecessary therapies.

THC2

- It shows mild thrombocytopenia with normal-sized platelets.
- Serious hemorrhage is rare.
- Megakaryocytes are present in bone marrow but are small and have hypolobulated nuclei (unlike CAMT where megakaryocytes are absent).
- No therapy is required. Again, recognition is important to avoid unnecessary therapies.

Congenital Amegakaryocytic Thrombocytopenia (CAMT)

- It has autosomal recessive inheritance and may have normal platelet number and function.
- Severe thrombocytopenia is seen in the neonates (<10,000/µl, MPV is normal) secondary to ineffective megakaryopoiesis.
- The disease is recognized during the first few days of birth due to easy bleeding/bruising.

Lab Diagnosis

- There is severe neonatal thrombocytopenia.
- Megakaryocytes are absent in the bone marrow.
- Diagnosis is confirmed by molecular analysis of the MPL gene.
- Most severe form of CAMT is due to complete absence of MPL receptor expression/function.
- Milder form is due to residual activity of a mutant MPL receptor.

Clinical Course

- Severe CAMT is recognized during the first few days of birth due to easy bleeding/bruising.
- Milder form is associated with low platelet number but sufficient to prevent severe bleeding.
- Mild form progresses to worsening thrombocytopenia by the first decade of life.
- It is also associated with reduced leukopoiesis and erythropoiesis.
- By the second decade of life it can progress to pancytopenia.
- When pancytopenia develops, CAMT may be confused with other marrow failure disorders like Aplastic anemia, Fanconi anemia and Dyskeratosis congenita.

Management

- Initial treatment consists of platelet transfusion in neonates to prevent serious bleeding.
- This will bring increments to platelet count and thus survival will be normal.
- Platelet transfusion should be reserved for symptomatic patients to prevent children from refractoriness because of alloimmunization.
- Hematopoietic stem cell transplantation (HSCT) should be considered as soon as the diagnosis is confirmed and the donor has been identified.

POINTS TO REMEMBER

- Over the last years, the pathogenesis of congenital platelet disorders has been better understood.
- The genes responsible for various congenital platelet diseases have been identified and advances are being made in the molecular characterization of these disorders.
- Careful collection of personal and family clinical data, an accurate physical examination and appropriate laboratory tests are invaluable for the evaluation of congenital platelet disorders.
- Using this approach, it is possible to identify the platelet defect correctly in some cases.
- However, the underlying molecular mechanisms remain unknown in most patients with a congenital bleeding disorder and impairment of platelet function.
- The challenge for the future is to increase our understanding of these diseases to obtain powerful strategies for prevention, diagnosis and therapy.

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29 Molecular Diagnosis of Thalassemia and Hemoglobinopathies

Sagir Akhtar

INTRODUCTION

- Hemoglobinopathies are disorders affecting the structure, function, or production of hemoglobin.
- Normal hemoglobin is a tetramer of two α and two β globin polypeptide chains.
- α-chain comprises 141 amino acids.
- β-chain comprises 146 amino acids.
- α-genes are located near the telomere of the short arm of chromosome 16 (16p13.3).
- β-genes are located on chromosome 11 (11p15.5) (Fig. 29.1).

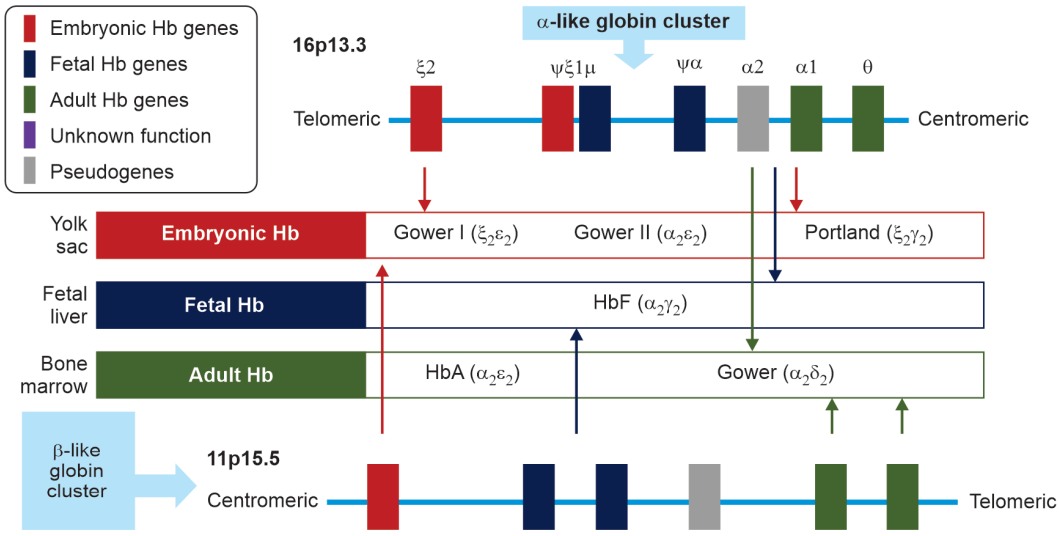

Fig. 29.1: Molecular structure of normal hemoglobin

- On chromosome 16, there are 3 genes within the α gene cluster, namely zeta (ζ), alpha 1 (α1), and alpha 2 (α2).
- On chromosome 11, there are 5 genes within the beta gene cluster, namely epsilon (ε), 2 gamma genes (γ), a delta gene (δ), and a beta gene (β).
- Duplicated α1 and α2 genes encode identical polypeptides.

- Fetal γ genes are duplicated (Gγ, Aγ) but encode globins differing only at amino acid 136.
- After 8 weeks of fetal life the embryonic hemoglobins, Gower-1 (ζ2ε2), Gower-2 (α2ε2), and Portland (ζ2γ2), are formed.

HEMOGLOBIN STRUCTURE

- At 9 weeks of fetal life, the major hemoglobin is HbF (α2γ2).
- HbA (α2β2) appears at ~1 month of fetal life but does not become the dominant hemoglobin until after birth, when HbF levels start to decline.
- Each globin chain enfolds a single heme moiety, consisting of a protoporphyrin IX ring complexed with a single iron atom in the ferrous state (Fe^{2+}).
- Each heme moiety can bind a single oxygen molecule.
- One molecule of hemoglobin can transport up to four oxygen molecules.
- Iron is coordinated to four pyrrole nitrogen of protoporphyrin IX, and to an imidazole nitrogen of a histidine residue from the globin chain.
- The sixth coordination position is available for binding with oxygen and other small molecules.

HEMOGLOBINOPATHIES CLASSIFICATION

They can be grouped into three main categories:

- Those owing to structural variants of hemoglobin, such as HbS.
- Those owing to failure to synthesize one or more of the globin chains of hemoglobin at a normal rate, as in the thalassemias.
- Those owing to failure to complete the normal neonatal switch from fetal hemoglobin (HbF) to adult hemoglobin (HbA) and this condition is known as a hereditary persistence of fetal hemoglobin (HPFH).
- An individual can also have a combination of two or more of these abnormalities

Thalassemia

- Thalassemia is an inherited blood disorder in which the body produces an abnormal form of hemoglobin which results in excessive destruction of red blood cells and further leads to anemia.
- The word thalassemia was first used in 1930.
- It derives from the Greek *thalassa* 'sea', and New Latin-emia (from Greek haima, *'blood')*
- It was coined because the condition called 'Mediterranean anemia' was first described in people of Mediterranean ethnicities.
- Classification of thalassemia is shown in Fig. 29.2.

TYPES

α-Thalassemia

- α-Thalassemia is caused by a deletion in approximately 95% of cases
- Minority of cases are due to point mutations
- Common mutations: - $\alpha^{3.7}$, - $\alpha^{4.2}$, -SA (South African deletion) and Hb constant spring

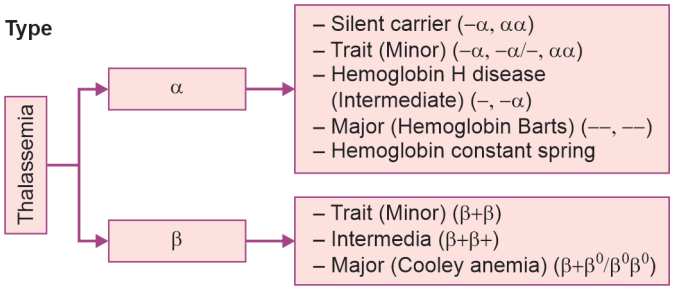

Fig. 29.2: Classification of thalassemia

- Hb Koya Dora (alpha142, Term-->Ser): Found at high frequency (10%) in certain communities of Andhra Pradesh.
- Hb constant spring (alpha142, Term-->Gln) and Hb Koya Dora: Termination codon mutations leading to an elongated alpha chain.
- The genetics of alpha thalassemia is shown in Fig. 29.3.

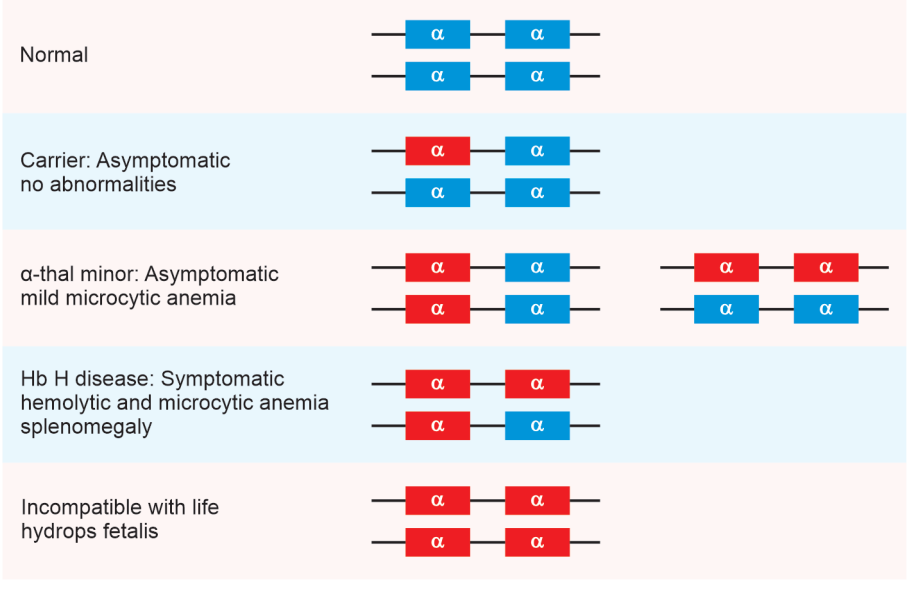
Normal
Carrier: Asymptomatic no abnormalities
α-thal minor: Asymptomatic mild microcytic anemia
Hb H disease: Symptomatic hemolytic and microcytic anemia splenomegaly
Incompatible with life hydrops fetalis
α

Fig. 29.3: Genetics of alpha thalassemia and its clinical consequences

β-Thalassemia

95% of β-thalassemias are due to point mutations that fall into either of 3 categories:

- Splicing mutations
- Promoter region mutations (reduce transcription)
- Chain terminator mutations.

Spectrum of β Thalassemia Mutations in India

- The average incidence of β-thalassemia trait in India is 3.3% with 1–2/1,000 couple being at risk of having an affected offspring each year.
- Common β thalassemia mutations include—***single base substitutions.***

- Over 200 mutations are seen but 5 common mutations in India: IVS 1–5 (G→C), IVS 1–1 (G→T), 619 bp deletion, codon 8/9 (+G), codon 41/42 (-TCCT).
- These 5 mutations alone account for around 89% of mutant chromosomes.
- Prevalence varies in different geographic regions.
- Considerable ethnic variations seen in Indians.
- 619 bp deletion in Sindhis, Lohanas (45–50%), Khoja and Memon Muslims from Gujarat (46%) and Punjabi Hindus (34%).
- IVS 1–5 (G→C) in Jains (94%), Brahmins, Patel and scheduled castes from Gujarat (58–78%), Brahmins and Marathas from Maharashtra (58–74%).
- IVS 1–1 (G→T) in Sindhis, Lohanas and Punjabi Hindus (25–32%).
- Codon 41/42 (–TCCT) in Baniyas from Gujarat (59%).

DIAGNOSTIC WORKUP

The detection and characterization of a hemoglobinopathy involves a three-tier workup:

- Full blood count
- Special hematological tests
- DNA testing.

DIAGNOSTIC MODALITIES

- Protein-based diagnostic modalities
- Molecular (DNA-based) methods
  1. Protein-Based Diagnostic Modalities
     - Cellulose Acetate Electrophoresis at Alkaline pH
     - Citrate Agar Electrophoresis at pH 6.0
     - HPLC
     - Isoelectric focusing
     - Capillary electrophoresis.
  2. Molecular (DNA-based) methods
     - Dot blot and Reverse Dot blot
     - Gap-PCR
     - MLPA
     - ARMS
     - RE-PCR.

DNA Diagnosis of Hemoglobinopathies

- The various types of thalassemia with the corresponding DNA diagnosis method is shown in Table 29.1.

Table 29.1: Various thalassemia disorders with the corresponding DNA diagnostic method

Disorder and mutation type	*Diagnostic method*
α^0-thalassemia	Gap-PCR, Southern blotting
α+-thalassemia: Deletion Non-deletion	 Gap-PCR, Southern blotting ASO, RE, DGGE

Contd.

Table 29.1: Various thalassemia disorders with the corresponding DNA diagnostic method	
Disorder and mutation type	***Diagnostic method***
β-thalassemia: Deletion Non-deletion	 Gap-PCR, ASO, ROB, ARMS, RE-PCR, DGGE
δβ-thalassemia	Gap-PCR, Southern blotting
HPFH Deletion Non-deletion	 Gap-PCR, Southern blotting ASO, ARMS, RE-PCR, DGGE
Hb Lepore HbS HbC HbE Hb D-Punjab Hb O-Arab Hb variants	Gap-PCR, ASO, RDB, ARMS, RE-PCR ASO, RDB, ARMS ASO, RDB, ARMS, RE-PCR ASO, RDB, ARMS, RE-PCR ASO, ARMS, RE-PCR RT-PCR and DNA sequencing

Collection of Blood and Preparation of Hemolysates

- EDTA is the most convenient anticoagulant because it is used for the initial full blood count and film.
- Although samples taken into any anticoagulant are satisfactory.
- Cells freed from clotted blood can also be used.

Preparation of Hemolysate for Qualitative Hemoglobin Electrophoresis

- Centrifuge EDTA blood at 3000–5000 rpm and remove plasma.
- Wash packed red cells with NS three times and remove supernatant as much as possible at the last washing round.
- Lyse 2 volumes of washed packed cells in 1 volume of distilled water and then add 1 volume of carbon tetrachloride (CCl4).
- Alternatively, lyse by freezing and thawing, then add 2 volumes of CCl4.
- Shake the tubes vigorously for approximately 1 min, then centrifuge at 1200 *g* (3000 rev/min) for 30 minutes at 4°C.
- Transfer the supernatant to a clean sample container and adjust the Hb to 100 ± 10 g/L with water.
- If an unstable Hb is suspected organic solvents should be avoided.
- Cellulose acetate electrophoresis at alkaline pH.
- Hemoglobin electrophoresis at pH 8.4–8.6 using cellulose acetate membrane is simple, reliable and rapid.
- It is satisfactory for the detection of the most common clinically important hemoglobin variants.

Principle

- At alkaline pH, hemoglobin is a negatively charged protein and when subjected to electrophoresis will migrate toward the anode (+).
- Structural variants that have a change in the charge on the surface of the molecule at alkaline pH will separate from HbA.

Interpretation

- Satisfactory separation of Hb C, S, F, A and J is obtained.
- In general, Hbs S, D and G migrate closely together, as do Hbs C, E and O^{Arab}.
- Differentiation between these hemoglobins can be obtained by using acid agarose gels, citrate agar electrophoresis, HPLC or IEF.
- All samples showing a single band in either the S or C position should be analyzed further using acid agarose or citrate agar gel electrophoresis, HPLC, or IEF to exclude the possibility of a compound heterozygote such as SD, SG, CE, or CO^{Arab}.

Electrophoresis Advantages

- Commercial, widely available, rapid methods used for many years.
- Gives an estimate of HbA2 level.
- Identifies some variant hemoglobins which are well characterized.

Electrophoresis Disadvantages

- Labor-intensive.
- Inaccurate in the quantification of low-concentration variants (HbA2) and in the detection of fast variants (HbH, Hb Barts).
- The precision and accuracy for HbA2 using scanning of electrophoretic gels is poor (in comparison to HPLC).

ALLELE-SPECIFIC OLIGONUCLEOTIDE (ASO) DOT BLOT

- Dot blot analysis is a technique for immobilizing amplified DNA on a nitrocellulose or nylon membrane.
- A large number of samples can be applied simultaneously on the surface of the membrane, enabling many different DNAs to be screened in a single hybridization.

REVERSE DOT BLOT (RDB)

- A technique for immobilizing allele-specific oligonucleotide probes on a nylon membrane rather than the individual DNA samples.
- Multiple pairs of mutant and normal ASO probes are spotted on strips of nylon membranes.
- The advantages and disadvantages of both these techniques are shown in Table 29.2.

Table 29.2: Advantages and disadvantages of ASO dot blot and reverse dot blot hybridization

Advantages	*Disadvantages*
⅄ ASO dot blot hybridization ★ Widely applicable and reliable	⅄ ASO dot blot hybridisation ★ Traditional protocols use radioactively labeled probes ★ Time-consuming and can only screen one mutation at a time ★ Expensive
⅄ Reverse dot blot hybridization (RDB) ★ Simultaneous screening for many mutations	⅄ Reverse dot blot hybridization (RDB) ★ Need sample controls to standardize new mutations ★ Need good technical expertise in the laboratory to set up and validate RDB ★ May be expensive if use commercial kits

Gap-PCR

- The most established approach.
- Principle is based on amplification using two primers complementary to the sense and antisense strand in the DNA region flanking the deletion.
- The advantages and disadvantages of this technique are shown in Table 29.3.

Table 29.3: Advantages and disadvantages of Gap-PCR

Advantages	*Disadvantages*
Simple, rapid and inexpensive	Need control DNA to validate test
Can be multiplexed to detect mutation	Limited to diagnosis of deletions with known DNA breakpoint sequences
Amplification of α-gene region technically difficult-possibility of allele drop-out	

Amplification-Refractory Mutation System (ARMS)-PCR

- It has been developed for the diagnosis of all the common β-thalassemia mutations found in all the main ethnic groups.
- Three primers are utilized as shown in Fig. 29.4.
- One primer is constant and complementary to the template in both reactions. The other primers are specific for wild and mutant types of genes of interest.

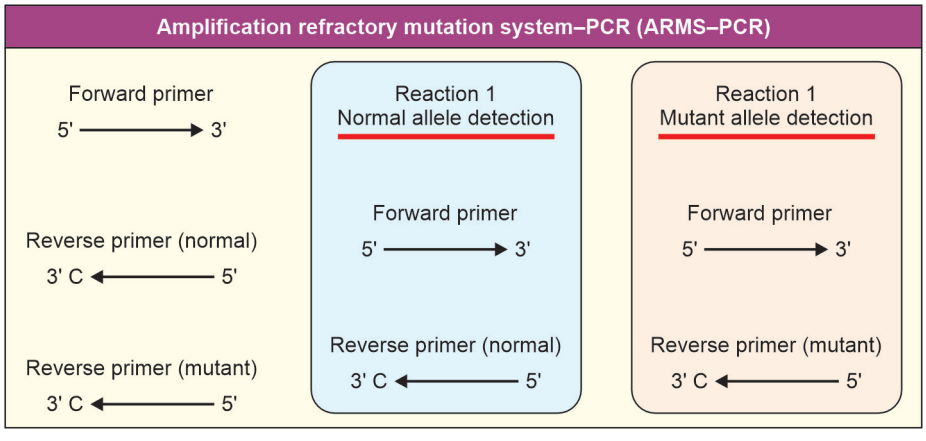

Fig. 29.4: Amplification-refractory mutation system (ARMS-PCR)

- If the sample is homozygous mutant or homozygous wild type amplification will only occur in only one of the tubes, if the sample is heterozygous amplification will be seen in both tubes.
- The advantages and disadvantages of this technique are shown in Table 29.4.

Table 29.4: Advantages and disadvantages of ARMS-PCR

Advantages	*Disadvantages*
Simple, rapid and inexpensive	Need control DNA to validate test and some rare mutations unavailable in homozygous state
Suitable for technical modification	Primers can degrade, giving non-specific signal
Can be multiplexed to detect mutation	

Multiplex Ligation-dependent Probe Amplification (MLPA)

- A variation of the multiplex polymerase chain reaction that permits multiple targets to be amplified with only a single probe pair.
- Each probe consists of two oligonucleotides which recognize adjacent target sites on the DNA.
- One probe oligonucleotide contains the sequence recognized by the forward primer, the other sequence is recognized by the reverse primer:
- LPO: Left probe oligonucleotide, LHS: Left hybridization sequence
- RPO: Right probe oligonucleoitde, RHS: Right hybridization sequence
- It consists of 3 steps:
  1. Denaturation and hybridization
  2. Ligation
  3. Amplification
- The forward primer used for probe amplification is fluorescently labeled, each amplicon generates a fluorescent peak which can be detected by a capillary sequencer.
- It is used for detection of an abnormal number of chromosomes, gene deletions, gene duplications, and gene expansions
- Limitation of this technique: MLPA requires the creation of labor-intensive probes for each new gene or chromosome.
- The advantages and disadvantages of this technique are shown in Table 29.5.

Table 29.5: Advantages and disadvantages of MLPA

Advantages	*Disadvantages*
Detects all deletion and duplication mutations (common, rare and novel) compared to gap-PCR	Does not provide a definitive diagnosis
Simple and rapid	Requires automated DNA sequencer

Restriction Fragment Length Polymorphism/RE-PCR

- It is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples.
- An RFLP probe is a labeled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis.

POINTS TO REMEMBER

- Hemoglobinopathies and thalassemias are a common cause of morbidity and mortality worldwide.
- One challenge in this area is that much of the disease burden of hemoglobinopathies and thalassemias is in parts of the world with limited resources for healthcare.
- Molecular diagnostic techniques are less commonly needed in the pediatric setting.
- Whereas these techniques are frequently critical in the setting of genetic counseling and prenatal diagnosis.
- The cost of these technologies will eventually decrease enough to make them available to resource-limited settings where the diagnosis of hemoglobin disorders will be most valuable.

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T Cell Immunotherapy for Hematological Malignancies

30

INTRODUCTION

- Immunotherapy is also known as biologic therapy.
- It is a type of cancer treatment designed to boost the body's natural defenses to fight cancer.
- It uses substances either made *in vitro* or *in vivo* to improve or restore immune system function.

HISTORICAL PERSPECTIVE

- In the 1980s, lymphokine-activated killer (LAK) cells were used to treat tumors in mice and humans.
- In the 1990s, cytokine-induced killer (CIK) cells amplified from peripheral blood mononuclear cells (PBMCs) were evaluated for cancer therapy.
- However, due to the lack of specificity, LAK and CIK-based therapy exhibited only limited efficacy.
- Allogeneic hematopoietic stem cell transplantation (HSCT) for hematological malignancies represented the earliest adoptive transfer of T cells with anti-cancer activity.
- Unfortunately, lack of specificity in the allogeneic responses made it challenging to separate the graft effect on tumor from the graft effect on host.
- In 2010 administration of lymphocytes genetically engineered to express a chimeric antibody receptor (CAR) against B cell antigen CD19 was shown to mediate regression of an advanced B cell lymphoma.
- In 2017, USFDA approved the first CAR T cell therapy Kymriah for children and young adults with Pre-B-ALL.

Types of Immunotherapy

- Monoclonal antibodies.
- Cancer vaccines
- Adoptive immunotherapy
- Nonspecific cancer immunotherapy
- Checkpoint inhibitors
- Oncolytic viruses.

- The various subtypes of each of these major types are shown in Fig. 30.1.

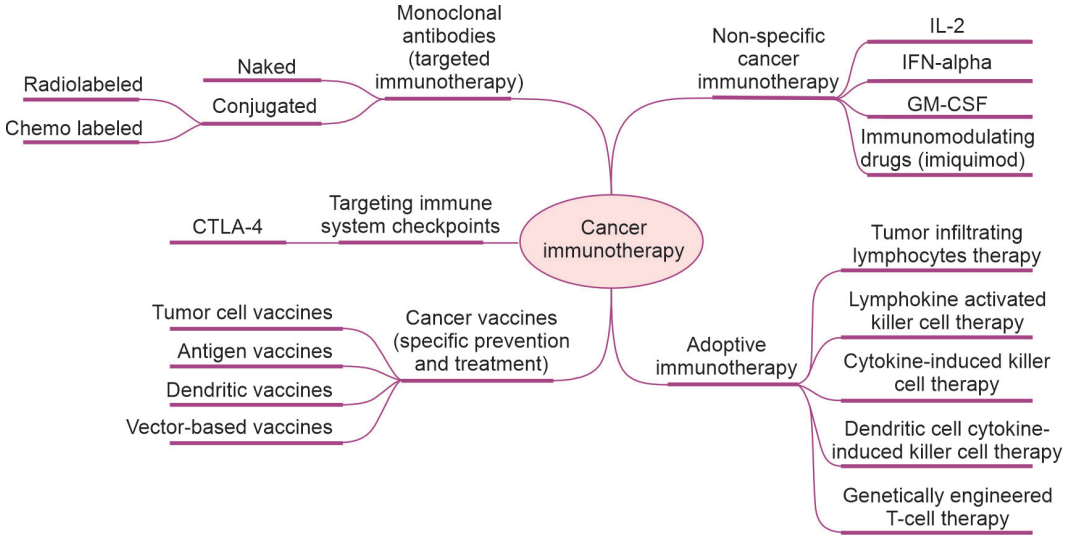

Fig. 30.1: Subtypes of major types of cancer immunotherapy

Monoclonal Antibodies

- These are antibodies that bind specifically to the tumor cells.
- A few examples are as follows:
  - *Alemtuzumab:* Naked humanized monoclonal antibody against CD52. It is used in cases of CLL.
  - *Brentuximab:* Chemolabeled chimeric antibody directed against CD30. Used on cases of Hodgkin's lymphoma and anaplastic large cell lymphoma.
  - *Ibritumomab:* Radiolabeled murine monoclonal antibody against CD20. It is used in cases of NHL.

Cancer Vaccines

- In cancer vaccines **tumor-associated antigens** are used and they are injected into cancer patients to induce a systemic immune response that may result in the destruction of the cancer cells.
- ***Tumor-associated antigens (TAA)*** are the mutated counterpart of normal proteins expressed by normal cells. These altered proteins which are expressed by tumor cells are known as tumor-associated antigens.
- ***Tumor-specific antigens (TSA),*** on the other hand, are antigens that are expressed by tumor cells only and not their normal counterpart cells.
- Certain tumor vaccines which are under clinical trial include:
  1. Vaccines against Wilms tumor-associated antigen-1 (WT-1) and proteinase 3 (PR3) in cases of AML, CML and MDS.
  2. Vaccine against modified synthetic BCR-ABL-derived breakpoint peptides (heteroclitic peptides) in CML.

T Cell Immunotherapy

- It predominantly comprises **adoptive immunotherapy.**
- In this patient's autologous T cells are expanded and manipulated *ex vivo*.
- These cells are then re-infused into the patient to exert anti-tumor response.

Modalities of Action

Following modalities exist for T cell immunotherapy:

- Cytotoxic T lymphocytes (CTLs)
- Antigen-specific autologous T cell clones
- Genetically engineered human lymphocytes.

Cytotoxic T lymphocytes (CTLs)

- The CTLs are an important subset of effector T cells.
- They act to mediate antitumor immunity by inducing cytolysis or apoptosis of malignant cells in a human leukocyte antigen (HLA)-dependent manner.

Procedure

- Tumor (at least 2 cm in size) is harvested by excisional biopsy.
- It is then dissected into fragments (1–3 mm^3).
- The dissected tissue is placed in wells with media.
- To this media IL-2 is added.
- Over the subsequent weeks tumor infiltrating lymphocytes (TILs) proliferate, while the adherent tumor cells die off or are killed by the lymphocytes.
- After 2–4 weeks of growth, TILs are tested for **T cell phenotype** and **reactivity to autologous tumor cells.**
- The wells containing cells with the **highest cytokine release** after co-culture with autologous tumor cells are selected and expanded for 2 weeks using a rapid expansion protocol.
- Antigen-specific T cells which can be detected in PBMCs of cancer patients.

TUMOR-SPECIFIC T CELL CLONES

- These are antigen-specific T cells that can be detected amongst the peripheral blood mononuclear cells (PBMCs) of cancer patients.
- They can be generated from repeated antigen-specific stimulation of T cells.
- There are two approaches that are available:
  - Using patient's own T cells (autologous) *in vivo*.
  - Donor-derived (allogenic) T cells *in vitro*.

GENETICALLY ENGINEERED T CELLS

These T cells are of two types:

1. T cells with chimeric antigen receptors (CARs).
2. T cells with T cell receptors (TCRs).

Advantages of T cell Immunotherapy

- T cell responses are specific and can thus potentially distinguish between healthy and cancerous tissue.
- These cells are quite robust and undergo up to 1,000-fold clonal expansion after activation.
- Traffic to the site of antigen, suggests a possible mechanism for eradication of distant metastases.
- They have memory thus, maintaining therapeutic effect for many years after initial treatment.

ROLE IN HEMATOLOGICAL MALIGNANCIES

- Hematological malignant cells utilize multiple pathways to evade cytotoxic T cell (CTL) mediated immunity and evolve resistance to currently available combinational therapies, resulting in relapse or failure of treatment.
- Broadly these mechanisms are of two types:
  - *Recognition dependent:* These mechanisms cause impaired recognition of tumor cells by CTLs.
  - *Recognition independent:* These mechanisms do not have any effect on the tumor cell recognition by CTLs.
- **Recognition dependent:** Following are the different mechanisms that are recognition dependent:
  1. Dysfunctional antigen-presenting cells (APCs) that fail to provide costimulation for priming tumor-specific CTLs:
     - Dendritic cells usually promote antitumor immunity via uptake and presentation of altered self-antigens or neoantigens from malignant cells.
     - However, it was observed that dendritic cells in patients with hematological are malignancies decreased in quantity and quality by tumor cells or other components of tumor microenvironment.
     - Tumor progression-related soluble factors like COX-2, PGE2, TGF-β and VEGF deregulate dendritic cell function which further impairs the presentation of tumor antigens.
  2. Defective antigen presentation of tumor cells in an HLA-dependent manner that contributes to the inability of CTLs to recognize malignant cells:
     - In hematological malignancies, it has been seen that the expression of HLA on the surface of tumor cells is **downregulated** as a result of mutations or deletions in the HLA loci.
     - Structural loss of HLA class I and II expression or mutations in HLA classes I and II loci have been reported to contribute towards immune evasion in **B cell lymphoma.**
  3. Low rates of mutational recognition in hematological malignancies compared to other malignancies:
     - Genome instability is generally a hallmark of tumor cells and can lead to somatic mutations that are entirely absent from the normal cells.
     - In contrast with other tumors—hematological malignancies show 10–20 times lower frequency of mutations.
     - Multiple myeloma contains ~3000 somatic mutations.

- AML, ALL and CLL each contain ~1500–2000 mutations.
- The lower mutational load in hematological malignancies likely relates to the inactive T cell response in the context of tumor progression.

- **Recognition independent:** Following mechanisms of evasion are independent of recognition of tumor cells:
  1. *Immune checkpoint pathways:*
     - Inhibitory pathways are critical for maintaining self-tolerance and modulating the immune response.
     - Two such major pathways exist—cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed-death 1 (PD-1) pathways.
     - Multiple myeloma, non-Hodgkin lymphoma, classic Hodgkin lymphoma and myelodysplastic syndrome escape host immunity by using both these pathways.
  2. *Regulatory soluble factors from suppressive immune cells:*
     - Suppressive immune cells, including regulatory T cells (Treg), tumor-associated macrophage (TAM), myeloid-derived suppressor cells (MDSC) secrete soluble factors.
     - These are inhibitory cytokines like IL-4 and IL-10.
     - They inhibit the response of leukemia-specific CTLs to the malignant cells.
- Hence, CTLs are considered to play a key role in antitumor immunity and because impaired recognition of CTLs to tumor cells contributes to immune evasion, regaining the ability of targeted recognition may be a critical component of targeted immunotherapy.
- Overcoming tumor immune evasion may be a critical event in the successful treatment of specific hematological cancers.
- In solid tumors such as melanoma, tumor-infiltrating lymphocytes isolated from tumor tissues have been shown to produce a partial antitumor effect.
- Despite similar success of allogeneic HSCT in treating or curing a majority of hematological malignancies, both allogeneic HSCT and adoptive transfer of tumor-infiltrating lymphocytes can lead to fatal complications or failure of treatment.
- This dilemma has prompted cancer immunologists to search for additional approaches to engineer CTLs to recognize and kill tumor cells specifically by counteracting tumor immune evasion.

Antigen-specific T cells

- Leukemic relapse after HSCT remains a major cause of treatment failure in high-risk patients who receive HSCT with poor prognostic characteristics.
- However, it was observed that patients who developed GVHD had reduced relapse rates, suggesting that lymphocytes present in engrafted cells can mediate a concurrent therapeutic graft versus leukemia effect.
- Since graft T cells are not selected for specificity for leukemia antigens, there is substantial morbidity and mortality from GVHD.
- It was, therefore, tried to enhance the GVL effect without promoting GVHD in post-transfusion patients.
- This is achieved by using specific CTLs directed against a particular tumor-specific antigen.

- For example, adoptive transfer of donor-derived ex vivo-expanded WT1-specific $CD8^+$ CTLs was done in post-transplant patients and initial results are promising.

T cells Directed Against Multiple Tumor-associated Antigens

- In this approach donor lymphocytes sensitized with multiple leukemia-associated antigens are used.
- This is done without exacerbating GVHD.
- It is mainly indicated for the treatment or prevention of leukemia relapse after HSCT.
- In one study CTL lines were manufactured from peripheral blood of 10 healthy donors by stimulation with the following five TAA:
  1. Proteinase 3 (Pr3)
  2. Wilms tumor gene 1 (WT1)
  3. Preferentially expressed antigen in melanoma (PRAME)
  4. Human neutrophil elastase (NE)
  5. Melanoma-associated antigen A3 (MAGE-A3)
     - It was seen that the multiple antigenic stimulation did not lead to inhibition of any single antigenic response and instead showed broad reactivity against myeloid malignancies.
     - Currently, a similar strategy using patient-derived CTLs targeting multiple lymphoma antigens is being evaluated clinically.
     - Adoptive therapy using non-gene modified T cells has been shown to be potentially effective, but on a limited basis currently, as clinical responsiveness remains low.

Limitations

- Despite a few successes, the broader application of this treatment modality is still greatly limited.
- The main reason for this is the lack of success with isolation and expansion of TSA/TAA-specific T cell clones.
- To achieve an optimal clinical outcome, it has become quite apparent that the following prerequisite conditions should be met:
  - An optimal target antigen should be chosen.
  - The quantity and quality of therapeutic T cells administered must be of guaranteed uniformity.
  - Tumor trafficking and *in vivo* persistence of infused T cells.

Genetically Modified T cells

- CARs are genetically modified receptors that are introduced and expressed in human T cells for targeting the surface antigens of tumor cells in their native conformation.
- They have **extracellular** single-chain variable fragments (ScFv) for antibody-like antigen recognition and **intracellular** signaling domains for activating T cells.
- In CAR-T cells, the extracellular domain ScFv is responsible for assigning the **specificity** of CTLs to the malignant cells.
- It can be designed according to specific antigens, e.g. against CD19 expressed in B cell ALL, CLL and lymphomas.

- In contrast, CAR intracellular signaling domains provide the necessary signals for **priming** T cell activation.
- In the CAR-T cell-mediated immune response, the ScFv of CARs directly bind to the tumor cells in an HLA-independent manner.
- Thus, specific binding of CARs with surface antigens leads to overcoming tumor immune evasion occurring due to impaired tumor antigen presentation.
- This thereby promotes the development of personalized CAR-T cell therapy.
- Currently, **CD19-targeted CAR** constructs have been used widely in relapsed B cell ALL, CLL and B cell NHL.
- Presently, three generations of CD19-targeted CAR-T cell-based adoptive immunotherapeutics have been used in clinical trials.
- The 'generations' of CARs typically correlate with the structure of **intracellular signaling domains.**
- The difference in various generations of CARs is illustrated in Fig. 30.2.

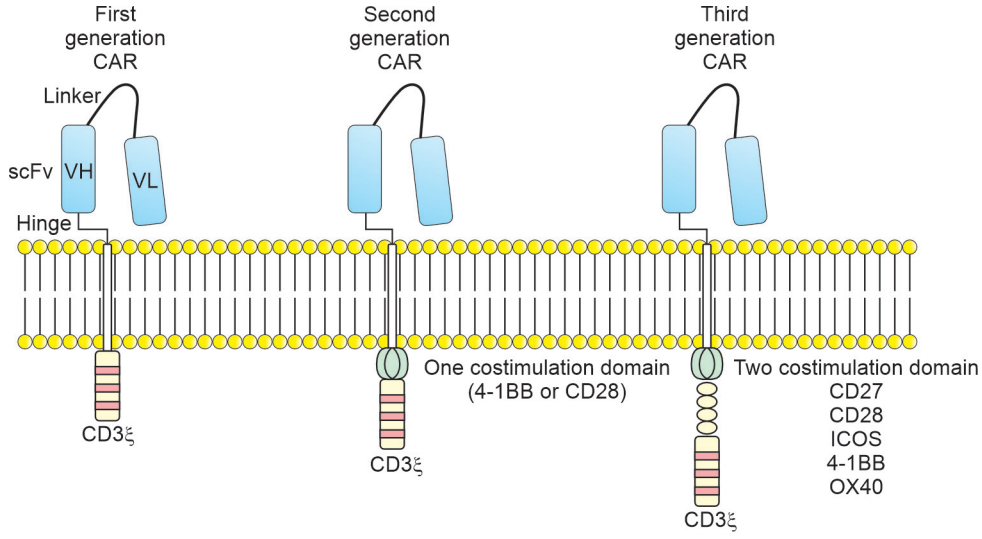

Fig. 30.2: Structures of various generations of chimeric antigen receptors

- Success of CD19-targeted CAR-T cell therapy is believed to be due to the fact that CD19 is expressed not only by leukemia cells but also by the **normal** antigen presenting B cells, which provides additional costimulatory signals for CAR-T cell activation.
- Secondly, this therapy produces clinically manageable symptoms.
- Recently, cancer-associated antigen **Tn glycoform of MUC1**-neoantigen expressed in a variety of cancers, was identified as a promising target that can be recognized by CAR-T cells.
- It was observed that anti-Tn-MUC1 CAR-T cells mediated target-specific cytotoxicity and successfully controlled tumor growth in xenograft models of T cell leukemia.
- In ongoing clinical trials surface antigens CD20, CD30, CD33, CD123, CD38, CD138, Ig κ light chain and Lewis-Y, have been selected as CAR targets in treating leukemia, lymphoma, and multiple myeloma.
- The results of these studies are awaited.

Disadvantages of CAR T cell Therapy

- These include systemic inflammatory response syndrome and neurological toxicities.
- **Systemic Inflammatory Response Syndrome:** It is also known as Cytokine release syndrome.
- It occurs hours to days after CAR-T cell adoptive transfer.
- There is the elevation of proinflammatory cytokines, T cell activation and expansion.
- Fever, malaise, myalgia, hypoxia, hypotension, renal dysfunction and coagulopathy are seen.
- Neurological toxicity manifests as headaches, confusion, ataxia, apraxia, facial nerve palsy, alterations in wakefulness, hallucinations, and dysphasia.

T Cell Receptor Transgene

- T cell receptors (TCRs) expressed on T cells are genetically engineered *in vitro* to specifically recognize and kill cancer cells.
- Two approaches are used for TCR gene transfer.
- In the first approach TCR specific to a tumor antigen is derived from either tumor infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) of the patient.
- In case of PBMCs the desired TCR is transduced into these cells using **lentiviral or retroviral vectors.**
- The cells are then amplified using a rapid expansion protocol.
- The amplified cells are infused into the patient along with IL-2.
- In the second approach generation of antigen-specific TCRs is done through the immunization of **HLA-I/II transgenic** mice with cancer antigens.
- The antigen-specific TCR is cloned and transduced into the peripheral blood T cells of the patient for immunotherapy.
- TCR-transduced T cells against several tumor antigens including MART1, CEA, gp100, NY-ESO-1 and MAGEA3 have been tested in clinical trials.
- The results from these clinical trials have shown great promise in treating various types of cancers including metastatic melanoma, metastatic colorectal cancer, metastatic synovial cell sarcoma and epithelial malignancies.
- In the context of hematological malignancy, two clinical trials of treatment for AML/MDS targeting WT1 are currently ongoing.

POINTS TO REMEMBER

- Hematological malignancies are known to show T cell evasion by various mechanisms.
- Newer techniques are increasingly being used for the treatment of these malignancies.
- Genetically modified CAR-T cell technique has shown promising results.
- However, the treatment modality is still in its infancy.
- The first CD19-CAR-T immunotherapy for B cell ALL has received FDA approval (Kymriah, Tisagenlecleucel) from Novartis.
- The second such approval has been given for the treatment of DLBCL Yescarta, axicabtagene ciloleucel from Kite Pharma.

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31 Targeted Therapy in Hematological Malignancies

Naveen Kumar

INTRODUCTION

- Targeted therapy has been defined as a type of treatment that blocks the growth of cancer cells by interfering with specific cell molecules required for carcinogenesis rather than by simply interfering with all rapidly dividing cells as with traditional chemotherapy.
- This '**magic bullet**' therapy is more effective and less harmful than systemic chemotherapy.
- The aims of targeted therapy are as follows:
  - To block specific pathways related to carcinogenesis and tumor growth
  - Inducing apoptosis of cancer cells
  - Blocking specific enzymes and growth factor receptors involved in cancer cell proliferation
  - Modifying the function of proteins that regulate cell proliferation

TYPES OF TARGETED THERAPIES

- Small molecules
- Monoclonal antibodies.

Small Molecules

- Small molecules are low molecular weight (less than 800 Dalton) organic compounds.
- They can penetrate the cell membrane and are designed to interfere with signaling pathways and to act on targets found inside the cell.
- The name of a drug provides a clue to the type of agent.
- Small molecules that have "-ib" as a suffix indicate a molecule that has inhibitory properties. Example: Imatinib.

Monoclonal Antibodies

- Monoclonal antibodies are designated humanized antibodies, which bind to cancer cell-specific antigens.
- Most monoclonal antibodies cannot penetrate the cell's plasma membrane and are designed against targets outside the cell or on the cell surface.

- Monoclonal antibodies have "-mab" as a suffix. Example: Rituximab targets CD20 on B cells.
- Another therapeutic application for these monoclonal antibodies is their use as a drug delivery system or antibody-drug conjugate (ADC).
- When a monoclonal antibody binds to cancer cells, the cytotoxic drug conjugated with the antibody is engulfed into the cancer cells.
- Thus, intracellular specific cell death is induced.
- This technology provides a wider therapeutic advantage.

TARGETED THERAPY IN MYELOPROLIFERATIVE NEOPLASMS

BCR-ABL POSITIVE MPN: CML

- Philadelphia chromosome-positive (Ph+) leukemias are a type of MPN that results from reciprocal translocation of a portion of chromosome 22 onto chromosome 9.
- This translocation leads to the fusion of the *BCR* gene to the *ABL1* proto-oncogene and the formation of the *BCR-ABL1* oncogene.
- CML was the first disease from where the era of targeted therapy started.
- Alois Matter, Jurg Zimmermann and Nick Lydon had synthesized a compound called GCP57148B now known as IMATINIB.
- In 1998 clinical trials started, within 6 months of clinical trials, all of the patients had their blood counts return to normal.
- Remarkably, this once-a-day pill had minimal side effects.
- Imatinib was approved by the USFDA in 2001, less than three years from the start of clinical trials.
- At diagnosis, 90–95% of cases of CML have the characteristic t(9;22) (q34.1;q11.2) reciprocal translocation that results in the Ph chromosome.
- This translocation fuses sequences of the BCR gene on chromosome 22 with regions of ABL 1 on chromosome 9.
- The site of breakpoint in chromosome 22 may influence the phenotype of the disease.
- In CML, the breakpoint cluster region (BCR) is almost always in the major BCR (M-BCR), spanning exons 12–16 (previously known as b1-b5).
- An abnormal fusion protein, p210, is formed, which has increased tyrosine kinase activity.
- Rarely, the breakpoint in the BCR gene occurs in the μ-BCR region, spanning exons 17–20 (previously known as c1-c4).
- A larger fusion protein, p230, is encoded.
- Patients with this fusion may demonstrate prominent neutrophilic maturation and/or conspicuous thrombocytosis.
- Although breaks in the minor breakpoint region, m-BCR (exons 1–2) lead to a short fusion protein (p190).
- It is most frequently associated with Ph chromosome-positive ALL.

Mechanism of Action of BCR-ABL1 Tyrosine Kinase Inhibitors

- The physiological binding of ATP to its pocket allows BCR-ABL1 to phosphorylate selected tyrosine residues on its substrates (Fig. 31.1).

- A synthetic ATP mimic such as Imatinib fits the pocket but does not provide the essential phosphate group to be transferred to the substrate.
- The downstream chain of reactions is then halted because with its tyrosines in the unphosphorylated form, the substrate does not assume the necessary conformation to ensure association with its effector.

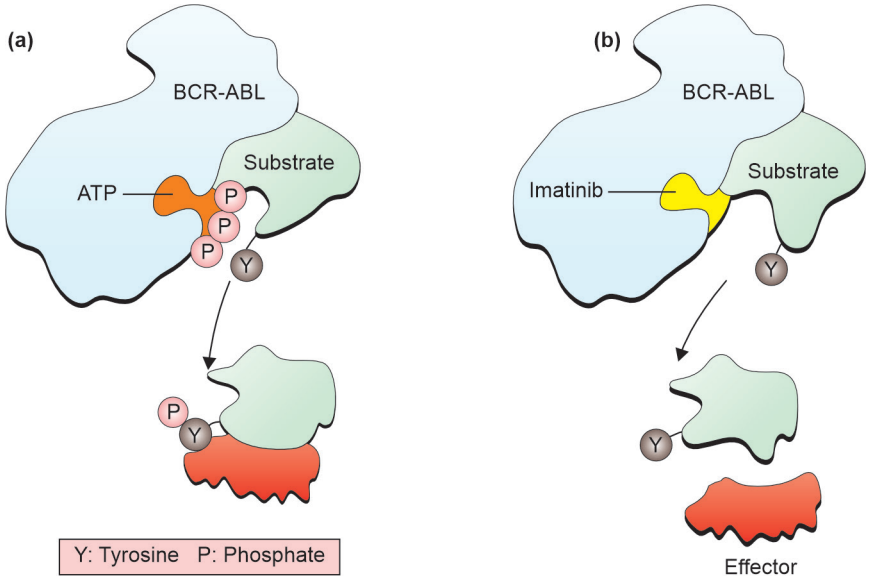

Fig. 31.1: Mechanism of action of BCR-ABL1 tyrosine kinase inhibitor

- The understanding of the abnormal signaling in CML cells led to the design and synthesis of small molecules that target the tyrosine kinase activity of BCR-ABL1.
- However, the emergence of subclones of leukemic progenitor cells that have BCR-ABL1 point mutations that alter amino acids and prevent the binding of the inhibitor to the BCR-ABL1 kinase domain can lead to drug resistance, particularly in the accelerated phase and blast phase.
- The second and third generations of TKIs (i.e. ***Nilotinib, Dasatinib, Bosutinib, and Ponatinib***) can circumvent this form of drug failure in the presence of most kinase domain mutations (Table 31.1).

Table 31.1: Comparison of tyrosine kinase inhibitors

	Imatinib (Gleevec)	*Nilotinib (Tasigna)*	*Dasatinib (Sprycel)*	*Bosutinib (Bosulif)*	*Ponatinib (Iclusig)*
Indications	First-line therapy (CP, AP, BP); relapsed/ refractory Ph+ ALL	First-line therapy (CP), resistance or intolerance to imatinib (CP and AP)	First-line therapy (CP), resistance or intolerance to other TKIs (CP, AP, or BP); Ph+ ALL with resistance or intolerant to prior therapy	Second-line therapy (CP, AP, BP with resistance or intolerance)	Resistance or intolerance to prior TKI or Ph+ ALL with resistant or intolerant to all other TKIs; all T3151 + cases

Contd.

Table 31.1: Comparison of tyrosine kinase inhibitors

	Imatinib (Gleevec)	*Nilotinib (Tasigna)*	*Dasatinib (Sprycel)*	*Bosutinib (Bosulif)*	*Ponatinib (Iclusig)*
Usual dosing	CP 400 mg/day AP/BP/ progression	CP 300 mg BID AP/BP 400 mg BID	CP 100 mg/day AP/BP 140 mg/ day	500 mg/day 600–800 mg/day	45 mg/day
Common toxicities (nonhemato-logic)	GI disturbance, edema (Including periorbital), muscle cramps, arthralgias, Hypophos-phatemia, rash	Rash, GI disturbances, elevated lipase, hyper-glycemia, low phosphorus, increased LFTs	Edema, pleural effusions, GI symptoms, rash, low phosphorus	GI (diarrhea), rash, edema, fatigue, low phosphorus, elevated LFTs	HBP, rash, GI, fatigue, headache

BCR-ABL1 Negative MPN

- BCR-ABL1 negative MPNs are a group of stem cell diseases, including Polycythemia vera, Essential thrombocythemia and Primary myelofibrosis.
- Currently, there is no curative therapy for these diseases other than bone marrow transplant.
- These MPNs are frequently associated with activating mutations in JAK2.
- The JAK 1/2 inhibitor ***Ruxolitinib*** was a major development, representing the first targeted treatment in this field.
- Despite a weak effect on the cause of the disease itself it improves the clinical state of patients and increases survival.
- In Ph-negative MPNs, the non-receptor tyrosine kinase JAK2, is frequently found to contain an activating V617F mutation.
- This mutation is present at a high frequency in patients with polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (MF) and infrequently other MPNs, MDS and AML.

The Janus Kinase 2 Gene

- The JAK2 gene is located on chromosome 9p24 in humans.
- It was named after the Roman God 'JANUS'. Four members have been identified: JAK1, JAK2, JAK3 and Tyk2.
- In 2005, the discovery of the JAK2 mutation (JAK2 V617F) expanded the knowledge about the pathogenesis of BCR-ABL negative neoplasms.
- This mutation results in the substitution of valine for phenylalanine at p.617 (JAK2 V617F) which is seen in approximately 95% in polycythemia vera, 50–60% in Essential thrombocythemia and 50–60% in primary myelofibrosis.
- JAK2 V617F mutation causes genetic instability in gene expression inducing changes in the chromatin structure and reducing the apoptotic response to DNA damage.
- Other new mutations in exon 12 were found in 2007.

- In 2013, mutations in CALR (calreticulin) were reported to be prevalent in ET and PMF patients with nonmutated JAK2 or MPL.

Mechanism of Action of JAK

- JAKs are associated with the intracellular domains of cytokine receptors.
- Intrinsic kinase activity mediated through their band-4.1 protein, ezrin, radixin, and moesin (FERM) and SH2 domains.
- It is kept in an inactive state in the absence of specific receptor-activating ligands.
- The signaling pathway is activated by one of the following cytokines like Interleukins (ILs), colony-stimulating factors (CSFs), Interferon (INF), Erythropoietin (EPO) and Thrombopoietin (TPO).
- Once the signaling pathway is activated, the cytokine binding leads to a conformational change of the receptor which causes activation and phosphorylation of JAKs.
- Mutations in JAK2 generate constitutive activation of the JAK/STAT pathway, mainly in STAT3 and STAT5.
- The STATs are located in the cytosol, phosphorylate and dimerize.
- The dimerized STATs migrate toward the nucleus, where they act as transcription factors which results in cell proliferation and survival.

Targeted Therapy

- ***Ruxolitinib*** has been approved by the USFDA for the treatment of myelofibrosis.
- ***Pacritinib*** and ***Momelotinib*** are under phase III clinical trials for polycythemia vera and essential thrombocythemia.

ACUTE LEUKEMIA

Acute Myeloid Leukemia

- AML is a genetically heterogeneous disease, characterized by accumulation of acquired genetic changes in hematopoietic progenitor cells.
- According to WHO the genetically-based classification provides the background for molecular targeted strategies.

Acute Promyelocytic Leukemia

- APML comprises 10–15% of acute myeloid leukemia.
- APML cases are characterized by a t(15;17) resulting in the formation of the PML-RARA fusion protein which causes arrest at the promyelocytic stage.

Mechanism of Action of ATRA

- ATRA treatment is characterized primarily by the induction of cellular differentiation rather than by cytotoxicity.
- ATRA induces APL blasts to undergo differentiation to the neutrophil stage.
- The USFDA approved ATRA for APML in November 1995.
- Recently Arsenic trioxide (Trisenox**)** which induces partial differentiation of promyelocytes has been approved by the USFDA in combination with the ATRA.
- It is for the treatment of newly diagnosed low-risk acute promyelocytic leukemia (APML) with the t(15;17) translocation or PML-RARA gene expression.

Recent Advances

- 2017 is called the landmark year for AML because after many years three targeted therapies have been approved by the USFDA.
  1. In April 2017, the first major breakthrough in AML therapy in many years was the approval of **Midostaurin** for FLT3-mutated AML
  2. In August 2017, **Enasidenib**, an IDH2 inhibitor, received US FDA approval for the treatment of AML.
  3. In September 2017, an antibody-drug conjugate (ADC) gemtuzumab ozogamicin (GO) was approved for CD33-positive AML.

FLT3 Inhibitors

- FLT3 is a cytokine receptor that is expressed almost exclusively in hematopoietic tissues.
- Its key roles are in early myeloid and lymphoid progenitor cell growth and survival.
- In AML, recurrent mutations commonly seen are as follows:
  - FLT3 internal tandem duplications (FLT3-ITDs) ~25% of patients.
  - Point mutations in the TK activation loop (FLT3-TKD) –5% to 10%.
  - They activate the kinase and promote growth, survival, and antiapoptotic signaling through MAPK, PI3K/AKT/mTOR, and STAT5 pathways.
  - Midostaurin is the first TKI to be approved in combination with standard intensive chemotherapy for adult patients with newly diagnosed FLT3-mutated AML.
  - The other FLT3 inhibitors under clinical trials are Quizartinib, Crenolanib and Gilteritinib.

IDH Inhibitors

- IDH1 mutations were first identified in AML in 2009.
- Isocitrate dehydrogenase is a ubiquitously expressed enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate.
- Due to IDH mutation 2-Hydroxyglutarate is formed which is an oncometabolite.
- This oncometabolite leads to epigenetic changes and impaired cellular differentiation.
- **Enasidenib** is an IDH2 inhibitor and emerged as the first IDH mutation-specific inhibitor in AML.
- Enasidenib induces terminal differentiation of leukemic blasts into neutrophils.
- The IDH 1 inhibitor which is under clinical trial is Ivosidenib.

CD33 Inhibitors

- CD33 has emerged as a logical target for leukemia therapy.
- CD33 expression is limited to myeloid progenitors and more differentiated blood cells.
- It is not present in normal hematopoietic stem cells.
- CD33 is expressed on leukemic blasts in >90% of AML patients.
- **Gemtuzumab ozogamicin** is an anti-CD33 antibody with calicheamicin (DNA intercalating) as its payload.
- In 2000, US FDA approved this drug for the treatment of relapsed AML cases.
- This was the first antibody-drug conjugate approved for cancer therapy and also the first targeted agent in non-M3 AML subgroups.

- In 2010, the FDA removed this from the market because of failure to confirm prior efficacy data and safety concerns.
- In 2017 it was reapproved with lower doses for relapsed and refractory CD33+ AML in adults and pediatric patients >2 years.

Mechanism of Action

- The antibody-drug conjugate (ADC) first binds to the tumor cell-specific antigen.
- The ADC-antigen complex undergoes endocytosis followed by degradation of ADC by lysosomal enzymes.
- After degradation the drug is released into the cytoplasm.
- The calechiamicin disrupts the DNA leading to tumor cell death.

TARGETED THERAPIES IN LYMPHOMAS

Hodgkin's Lymphoma

- Classical Hodgkin lymphoma represents ~10% of all lymphomas.
- Treatment for relapsed and refractory disease is chemotherapy followed by autologous stem cell transplant (ASCT) with ~ 50% cure rate.
- Several studies show that achieving a complete response prior to ASCT is one of the most important factors in determining long-term outcomes post-ASCT.

Targeted Therapy

1. **Anti-CD30 Antibody:**
   - In 2011 FDA approved **brentuximab vedotin**, an anti-CD 30 antibody-drug conjugate.
   - This drug was the first major advance in the management of Hodgkin's Lymphoma after several decades in patients with refractory disease or relapse.

   Mechanism of action
   - Anti-CD30 antibody is conjugated to the potent anti-microtubule agent monomethyl auristatin E (MMAE).
   - After binding to CD30 the ADC Antigen gets rapidly internalized and transported to lysosomes followed by degradation of ADC by lysosomal enzymes.
   - Then the anti-microtubule agent monomethyl auristatin E (MMAE) is released and binds to tubulin, leading to cell cycle arrest and apoptosis.
2. **PD1/PDL1 inhibitors**
   - PD-1 is expressed on T cells, B cells, NK cells and many tumor-infiltrating lymphocytes (TILs).
   - PD-1 binds two ligands PD-L1 and PD-L2.
   - In the tumor microenvironment, PD-1 and its ligand PD-L1 perform a vital role in tumor progression and survival by escaping tumor neutralizing immune surveillance.
   - The function of a tumor overexpressing PD-L1 is to protect itself from cytotoxic T cell (CD8+) mediated cell killing.
   - Among hematological malignancies, PD-1/PD-L1 inhibitors have been quite successful in the treatment of classical Hodgkin lymphoma.

- PD-L1 and PD-L2 are expressed on the surface of malignant cells in 65–100% of classical Hodgkin's lymphoma and 54% nodular lymphocyte predominant Hodgkin's lymphoma.
- The reason for this is an amplification of the chromosomal region 9p24.1 which contains the genes encoding PD-1 and JAK2.
- It typically exhibits an over-expression of PD-1 ligands due to alterations in chromosome 9p24.1.
- This results in increased expression of proteins on RS cells.
- JAK2–STAT signaling promotes further increased expression of PD-1 by augmenting transcription of the PD1 gene.
- The PD-1 inhibitors approved by USFDA for classical Hodgkin's lymphoma are **nivolumab** and **pembrolizumab.**

Non-Hodgkin Lymphoma

- NHL is the most common hematologic malignancy.
- The USFDA-approval of anti-CD20 monoclonal antibody rituximab in 1997 revolutionized treatment of B cell NHL.
- This changed the standard therapy from chemotherapy to combination chemoimmunotherapy.

Rituximab

- It is a monoclonal antibody directed against the CD20 antigen which is a transmembrane protein located on normal pre-B and mature B lymphocytes.
- Rituximab is used as first-line treatment in CD20-positive diffuse large B cell lymphoma along with chemotherapy (R-CHOP).
- It is also used as first-line therapy in CD20-positive low grade or follicular B cell NHL along with chemotherapy.

Newer Anti-CD20 Antibody

1. **Ofatumumab**
   - This is a high-affinity IgG1 monoclonal antibody directed against CD20 which acts by triggering complement-dependent cell lysis (CDCL) and antibody-dependent cell-mediated cytotoxicity (ADCC) of B cells over expressing CD20.
   - Ofatumumab is approved for chronic lymphocytic leukemia as first-line treatment in combination with chlorambucil.
2. **Obinutuzumab**
   - This is a 3rd generation anti-CD20 antibody which acts by enhanced antibody-dependent cellular cytotoxicity (ADCC).
   - It is used in untreated CLL in combination with chlorambucil.
   - It is used as 2nd line treatment in combination with bendamustin for follicular lymphoma which was previously treated with rituximab and failed.
   - It is an orphan drug for marginal zone splenic B cell lymphoma as well.
3. **Ibritumomab tiuxetan**
   - Ibritumomab is a murine IgG1 kappa monoclonal antibody directed against CD20.
   - It is bound to indium (In111) or yttrium (Y 90).

- It specifically delivers a potentially cytotoxic dose of radiation which is known as radioimmunotherapy.
- This is indicated in relapsed or refractory low-grade, follicular, or transformed B cell NHL and also for rituximab refractory follicular NHL.

Anti-CD30 Antibody-drug Conjugate

- Brentuximab vedotin is a CD30-directed antibody-drug conjugate.
- This is indicated in systemic anaplastic large cell lymphoma after the failure of at least one multi-agent chemotherapy regimen.

Proteasome Inhibitor

- Bortezomib is a proteasome inhibitor.
- It reversibly inhibits the 26S proteasome complex that degrades ubiquitinated proteins.
- It acts by blocking the targeted proteolysis which disrupts cell signaling pathways leading to cell cycle arrest, apoptosis and inhibition of angiogenesis.
- Bortezomib also inhibits nuclear factor NFkβ-mediated cell survival and tumor growth.
- It is used in the treatment of Multiple myeloma and also in the treatment of mantle cell lymphoma with failure of chemotherapy.
- Carfilzomib is another proteasome inhibitor that is under clinical trials.

Kinase Inhibitors

- Ibrutinib is a small-molecule inhibitor of tyrosine kinase which irreversibly inhibits TK activity.
- It leads to an inhibition of the growth of malignant B cells.
- This is used as 2nd line therapy in mantle cell lymphoma who have received at least one prior therapy and in CLL who have received at least one prior therapy.
- Sunitinib, dasatinib and sorafenib are under clinical trials.

Histone Deacetylases (HDACs) Inhibitors

- Vorinostat is a synthetic hydroxamic acid derivative with anti-neoplastic activity by inhibiting deacetylation.
- It leads to the accumulation of both hyperacetylated histones and transcription factors.
- Hyperacetylation of histone proteins results in the upregulation of cyclin-dependant kinase p21, followed by G1 arrest.
- Hyperacetylation of non-histone proteins such as tumor suppressor p53, alpha-tubulin and heat-shock protein 90 produces additional anti-proliferative effects.
- It is used in the treatment of cutaneous T cell lymphoma which should be either a recurrent disease or following two systemic therapies.
- Belinostat is another HDAC inhibitor with anti-neoplastic activity which is used in relapsed or refractory peripheral T cell lymphoma (PTCL).

Cytokine Therapy

- Denileukin diftitox is a cytotoxic recombinant protein consisting of interleukin-2 (IL-2) protein sequences fused to diphtheria toxin.

- Denileukin Difitox is a CD25-directed cytotoxin.
- The cytocidal action of diphtheria toxin on the cells that express IL-2 receptors is mainly due to ADP-ribosylation of EF-2 causing inhibition of mRNA prolongation and ultimately inhibition of protein synthesis.
- It is used in the treatment of persistent or recurrent cutaneous T cell lymphoma.

Interferon Alfa-2b

- A non-glycosylated recombinant interferon with antiviral and anti-neoplastic activities.
- Alfa interferons bind to specific cell-surface receptors, resulting in the transcription and translation of genes leading to protein products that mediate antiviral, antiproliferative, anticancer and immune-modulating effects.
- It is used in Hairy cell leukemia patients who are 18 years of age or older.
- It is also used in clinically aggressive follicular NHL along with chemotherapy for patients 18 years of age or older.

Immunomodulators

- Lenalidomide is a thalidomide analogue with potential antineoplastic activity.
- It stimulates T cells and reduces serum levels of VEGF and FGF and inhibits angiogenesis.
- This agent also promotes G1 cell cycle arrest and apoptosis of malignant cells.
- Lenalidomide is used in relapsed mantle cell lymphoma or progressed after two prior therapies.
- It is also used in multiple myeloma as maintenance therapy in patients who received an autologous stem cell transplant.

Mammalian Target of Rapamycin (mTOR)

- mTOR is an intracellular serine/threonine kinase that exists as a downstream component of numerous signaling pathways.
- The activation of mTOR results in the production of proteins involved in cell metabolism, growth, proliferation and angiogenesis.
- Currently no mTOR inhibitor is approved by USFDA for the treatment of NHL.
- Temsirolimus and everolimus are the mTOR inhibitors under clinical trials.

POINTS TO REMEMBER

- The discovery of specific genetic alterations has increased our understanding of pathogenesis and revolutionized therapeutic strategies.
- As we begin to understand the key signaling pathways and molecular drivers, targeted therapy will continue to develop.
- Targeted therapy is a type of treatment that blocks the growth of cancer cells by interfering with specific cell molecules required for carcinogenesis.
- It doesn't just simply interfere with all rapidly dividing cells as with traditional chemotherapy.
- The clear fact is that pathology has a central role in determining treatment choice and future advances in targeted therapies.

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Section

4

Transfusion Medicine

Cord Blood Banking

32

Sakshi Sirswal

INTRODUCTION

- Cord blood is the blood remaining in the umbilical cord and placenta after birth.
- It is rich in stem cells.
- After the baby is born, the umbilical cord is clamped and cut.
- The umbilical cord blood is sampled from the cord.

UMBILICAL CORD AT A GLANCE

- During prenatal development, the umbilical cord is physiologically and genetically part of the fetus.
- The umbilical cord has two umbilical arteries and one umbilical vein within Wharton's jelly.

HISTORY

- In 1974, umbilical cord blood (UCB) was reported to be a source of hematopoietic stem and progenitor cells.
- In 1988, the first transplantation of cryopreserved UCB to a six-year-old male child with Fanconi anemia, an inherited bone marrow disease, was performed in France.
- Over the next 30 years, numerous studies were published demonstrating the regenerative potential of particular UCB-derived cell populations.
- A global network of public and private biobanks of UCB was established.
- In 1992, First public bank for umbilical cord blood was established by Dr. Pablo Rubinstein at the New York Blood Center through funding provided by the National Heart, Lung and Blood Institute (NHLBI) of the National Institutes of Health (NIH).

Advantages of Cord Blood

- It is a readily available source of hematopoietic stem cells.
- It is an alternative to bone marrow or peripheral stem cell transplantation to treat malignant and non-malignant conditions in children and adults.
- Lower incidence and severity of graft versus host disease.
- Lower incidence of viral transmission: in particular, cytomegalovirus and Epstein-Barr virus.

- Faster availability-patients on average receive cord blood transplantation earlier than those receiving conventional bone marrow grafts.
- It is harder to collect bone marrow than it is to collect cord blood.
- Collecting bone marrow poses some risks and can be painful for the donor.
- Cord blood transplantation tolerates a greater degree of mismatch of tissue types between the donor and the recipient greater than is acceptable with bone marrow or peripheral blood.
- Because of the ethnic diversity of donors of cord blood, there is a higher frequency of non-Caucasoid HLA haplotypes available compared with bone marrow registries.
- Bone marrow donors may change their minds over time or may no longer be available.
- In case of related donors, HLA-identical sibling cord blood transplantation has been performed almost exclusively in children.
- The lower risk of both treatment-related mortality and chronic graft versus host disease makes cord blood transplantation a particularly successful option for children with hemoglobinopathies.
- Cord blood transplants from unrelated donors for children have been associated with sustained engraftment, a low incidence of graft versus host disease and no higher risk of leukemic relapse.
- There is a theoretical hope that stem cells will be a crucial part of future treatments for Diabetes, Alzheimer's, spinal cord injuries, heart failure, stroke, etc.

Disadvantages of Cord Blood

- Low numbers of hemopoietic progenitor cells and stem cells in each cord blood donation, may cause delayed engraftment.
- Cord blood has a limited number of stem cells, not enough to treat most adults.
- Lack of availability of subsequent donations of stem cells and/or lymphocytes from the graft donor in graft failure or disease relapse.
- Own cells may be inappropriate in genetic conditions including some leukemias, because they may carry leukemogenic mutations.
- Only half to a third of samples yield mesenchymal stem cells and clinical uses for mesenchymal cells are speculative.
- Child may never need it and possible benefits are too remote to justify the costs.

HOW TO COLLECT CORD BLOOD?

- There are two methods: Open system and Closed system.
- Both procedures involve a direct puncture of the umbilical vein under sterile conditions.
- Open system involves sampling by needle and syringe.
- Closed system involves the collection of the umbilical cord in a blood collection bag.

i. **Cord blood collections**

a. Prerequisite for collection is the availability of trained staff for:

i. Collecting the cord blood

ii. Mother's blood sample

iii. Obtaining their medical and family histories

iv. Consent for donation.

b. Cord blood can be donated only if the mother and infant are eligible.

- Contraindications
  - Mother suffering from infectious disease during pregnancy.
  - Genetic disease in the infant that might get transmitted to the transplant recipient.
  - Family history of transplantable genetic disease that might be present in the infant.
  - The pregnancy was the result of an ovum donation, or a sperm donation from a man other than the husband.
  - The mother has a history of leukemia or other cancer.
- Collection can be done either after cesarean section or vaginal delivery.
- While the placenta is *in utero* or after the removal of placenta from the mother.
- A sterile blood collection bag set with attached tubing and needle is used.
- The cleaned surface of the cord is punctured with the needle, to enter the umbilical vein.
- The cord blood drains through the tubing into the bag by gravity.
- 70–150 mL of blood is collected in a bag containing 100 mL of ACD/CPD.
- A unique identification (ID) number is assigned, using a pre-printed bar-coded label.
- Data forms and all specimen tubes get identical ID labels, both bar-coded and human-readable.
- Testing of maternal blood for Syphilis, HBsAg, anti-HIV, anti-HCV, anti-HBc.
- The cord blood at the time of collection is tested for any bacterial or fungal contamination.
- CD34+ cell analysis is done for progenitor cell content.
- ABO and Rh typing of cord blood.
- HLA typing of the cord blood is done.
- Samples are saved and may be used for further testing in the future.

HLA TYPING

- HLA typing can be done by the following methods:

i. **Serology**
  - Used to be the gold standard before the advent of more advanced molecular methods.
  - It is an antibody-based histocompatibility testing.
  - Low-resolution typing.
  - Identifies molecules that are actually expressed on the cell and are involved in the rejection of the mismatched grafts.

ii. **Mixed lymphocyte culture**
  - It is rarely being used now.
  - Originally it was used for class II typing.
  - It involves culturing together lymphocytes of two individuals where each cell population is able to recognize the 'foreign' HLA class II antigens of the other.

iii. **Molecular assays**
  - It is DNA-based histocompatibility testing.
  - It is rapidly becoming the method of choice.
  - Intermediate and high-resolution typing
  - Identifies different nucleotide polymorphisms which code for the different allelic variants.

Serological Typing

- Lymphocytes are HLA-typed by cross-matching to panel reactive antibodies (PRA) using the complement-dependent cytotoxicity (CDC) test.

MOLECULAR TYPING

The molecular methods include the following techniques:
- SSP-PCR
- SSO-PCR
- DNA sequencing.

Sequence-specific Primers/SSP-PCR

- The principle of this method is that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or several mismatches.
- Specificity is determined by the use of sequence-specific primers in which a 3′ single-base mismatch inhibits the priming of non-specific reactions.
- Because Taq polymerase lacks 3′ to 5′ exonuclease activity, even if primer pairs do anneal non-specifically, they will not amplify efficiently.
- Thus only the desired allele or alleles will be amplified and the amplified product can then be detected by agarose gel electrophoresis.

Sequence-specific Oligonucleotides/SSO

- Hybridization of PCR-amplified DNA with sequence-specific oligonucleotide (SSO) probes was the first molecular typing method used to detect HLA Class II alleles.
- Specificity for a particular HLA locus was achieved by selecting PCR primers specific for a sequence in the conserved region of the second exon.
- Methods in the clinical laboratory have tended to use either a substrate in a staining development system or a substrate that generates a chemiluminescent signal.

PROCESSING

- Presently, the vast majority of cord blood collections are red blood cell (RBC) reduced prior to cryopreservation.
- Several methods are in use to accomplish this goal, including Hespan sedimentation to obtain a modified buffy coat, density gradient centrifugation to obtain enriched mononuclear cells (MNC), and 2 automated processes [Sepax and automated processing platform (AXP)] that result in a buffy coat product.
- The Hespan, Sepax, and AXP processing methods result in cord blood products composed of all nucleated cell populations found in the original collection [Mononuclear cell (MNC), neutrophils, some RBCs].
- Whereas the Ficoll method enriches the stem-cell-containing MNC subpopulation (generally greater than 85% MNC with a few contaminating neutrophils and nucleated RBC).
- Before and after processing, small samples are removed to count the total nucleated cells (TNC) and the number of nucleated cells that are positive for CD34.
- The freezing bag contains the 20 mL final product.

FREEZING AND STORAGE

- Cold, sterile, Dimethylsulfoxide (DMSO, 5 mL of a 50% solution) is added slowly to the freezing bag.
- **Quarantine:** Before freezing, each cryoprotected cord blood unit is sealed in a Teflon overwrap bag that insulates and quarantines each unit individually within the freezer, preventing possible contamination from infected units, plastic surfaces and from organisms 'swimming' in the liquid nitrogen.
- **Controlled rate freezing:** Cools down to –50°C slowly at the rate of 2.8°C/minute followed by freezing at –196°C in liquid nitrogen.

TESTING OF CORD BLOOD UNITS

Following tests are carried out on each cord blood sample.

- HLA typing
- Cell counts: Total and mononuclear cell concentration, used to calculate cell dose for each product.
- CD34 analysis by flow cytometry.
- Colony-forming cell cultures
- Bacterial and fungal cultures
- Cell viability assay.

SHIPMENT

After one or more cord blood units have been selected for transplantation, the transplant center may request shipment, on a specific date, scheduled to arrive before the patient begins conditioning to prepare for the transplant.

APPLICATIONS

- Stem cells derived from umbilical cord blood can be used to treat 80+ diseases including leukemia, lymphoma and more.
- 1 in 3 people is estimated to benefit from regenerative medicine including cerebral palsy and autism, require patient's own cord blood.

DIFFERENCE BETWEEN PUBLIC AND PRIVATE BANKS

- **Private cord blood banks:** Costs $1000–2000 initially+ $100/year; Ownership of the blood retained by the family.
- **Public cord blood banks:** Cost: free. Blood donated and not reserved for child or family. May be used for research in some instances (after informed consent).
- **Cord blood banks in India:**
  - Reliance Life Science—first public CB bank (2002)
  - Life Cell—India's first private cord blood bank (2004)
  - Jeevan Stem Cell Bank, public as well as private
  - Cord blood prices in India: Minimum 70,000 to a maximum of 1,50,000 in private blood banks.

CORD BLOOD BANKING GUIDELINES

- The Drug Controller General of India (DGCI) within CDSCO established guidelines for cord blood banking, which became effective in 2012.
- Applicable to cord blood units intended for autologous and allogeneic use and are designed to provide a framework for facilities to obtain a license to manufacture and distribute cord blood in India.

Some transplant centers in India:

- Tata Memorial Hospital, Mumbai
- Adyar Cancer Centre, Chennai
- Apollo Speciality Hospital, Chennai
- Apollo Hospital, Hyderabad
- Christian Medical College, Vellore
- R&R Army Hospital, New Delhi
- AIIMS, New Delhi
- Armed Forces Medical College, Pune
- Sanjay Gandhi PG

POINTS TO REMEMBER

- A cord blood bank is a facility which stores umbilical cord blood for future use.
- Both private and public cord blood banks have developed in response to the potential for cord blood in treating diseases of the blood and immune systems.
- Public cord blood banks accept donations to be used for anyone in need, and as such function like public blood banks.
- Traditionally, public cord blood banking has been more widely accepted by the medical community.
- Policymakers should therefore promote public cord blood banking.
- The narrow indications of the possible utility of the baby's cord blood stored privately should be highlighted to the family opting for cord blood banking.

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Section

5

Clinical Pathology

Hepatitis E—Recent Advances

33

INTRODUCTION

- Hepatitis E is an important cause of non-A/non-B hepatitis.
- It was first suggested as a causative agent for an outbreak in Kashmir in 1978.
- In this outbreak Hepatitis A and Hepatitis B viruses were not detected and another hepatotropic virus was suggested as a causative factor.
- The stool samples from said patients were analyzed using electron microscopy.
- Markers of acute hepatitis A and B infection were not detected.
- However, virus-like particles were visualized.
- In 1983 small virus particles were identified in Soviet soldier's stool samples by electron microscopy.
- In 1989 the viral agent was given the name of HEV in Tokyo International Conference.
- In 1990 the virus was cloned and the first serological test was developed in 1991.

CLASSIFICATION

- Family: Hepeviridae
- Genus: Orthohepevirus
- Species: Orthohepevirus A
- It has a total of seven genotypes out of which four genotypes infect humans.
- Genotype 1: Five subtypes
- Genotype 2: Two subtypes
- Both these genotypes only infect humans.
- Genotypes 3 and 4:10 and 7 subtypes respectively. They have been isolated not only in humans but also in animals in both developing and industrialized countries.
- Genotypes 3 and 4 have a zoonotic nature but can infect humans.
- Genotypes 5 and 6 have a strictly zoonotic nature and do not infect humans.
- Recently Genotype 7 has also been found to infect humans.
- Hepatitis E virus is the only hepatotropic virus that has animal reservoirs.

STRUCTURE

- It is a nonenveloped virus.
- There is presence of icosahedral symmetry.

- The viral particles can have two different sizes, i.e. 32 and 34 nm.
- These are 6.6 and 7.3 kb in length respectively.
- HEV has a single-stranded positive polarity ribonucleic acid (RNA) molecule.
- There are three open reading frames (ORFs) in the viral genome.
- A novel ORF 4 has been recently reported.

ORF 1

- **Length** is 5082 nucleotides.
- **Location** is near 5′ end after 25 non-coding regions.
- **It encodes** 1693 amino acid long polyprotein for replication and protein processing through RNA-dependent RNA polymerase.
- **There are eight domains as follows:**
  - Met—Methyltransferase enzyme.
  - Y domain—Unknown function.
  - Papain-like protease—Polyprotein processing.
  - HVR domain—Unknown function.
  - Proline rich polyproline region—For RNA replication.
  - Macro-domain—Inhibits NF-κB.
  - Helicase—Unwinds RNA before replication.
  - RNA-dependent RNA polymerase—RNA replication.

ORF 2

- **Length** is 1983 nucleotides.
- **Location** is near 3′ end.
- **It encodes** 660 AA protein for viral capsid.
- This protein is involved in attachment to host cells and induction of neutralizing antibodies.
- Three glycosylation regions, divided into three domains have been identified in ORF2, but the **biological relevance** of these potential modifications is unclear.
- However, it has been observed that mutations within the glycosylation sites prevent the formation of infectious virus particles.
- These 3D structure studies reveal that the HEV capsid is formed by capsomeres consisting of homodimers of a single capsid protein forming the virus shell.
- Thus, it is plausible that glycosylation is an important step in viral particle synthesis.

ORF 3

- **Length** is 300 nucleotides.
- **Location** overlaps with ORF 2 only.
- **It encodes** a small immunogenic phosphorylated protein (pORF 3) involved in virion morphogenesis and release.
- The overlapping part with ORF 2 is the most conserved region of the viral genome.
- All ORFs are expressed during viral infection.
- This is because antibodies against these regions have been detected in naturally infected humans and in experimentally infected monkeys.
- Additionally, there are two untranslated regions (UTRs) in 3′ and 5′ terminal portions.

NOVEL ORF 4

- **Length is** 2385–3308 nucleotides.
- It has been only identified in Genotype 1.
- ORF 4 product interacts with multiple viral proteins to form a protein complex consisting of viral RdRp, helicase and X, and promotes viral replication.

EPIDEMIOLOGY

- In developing countries, it mainly spreads via contaminated water (Genotypes 1 and 2).
- In contrast in developed countries animal reservoir is responsible (Genotypes 3 and 4).
- The seroprevalence in industrialized countries is greater than it was previously thought when compared to the number of cases reported in the native population.
- This theory is further strengthened by studies from Japan that have phylogenetically linked viruses recovered from uncooked meat (pigs and deer) and humans who became infected after consuming the meat.

STATISTICS

- Incidence is 20 million HEV infections worldwide with 3.3 million symptomatic cases.
- 44,000 deaths (accounting for 3.3% of the mortality due to viral hepatitis) occur annually.
- Seroprevalence in industrialized countries ranges from 1 to 5%.
- Highest prevalence has been reported from East and South Asia (Seroprevalence –10–50%).
- The overall mortality is 1%.
- The HEV outbreaks occur either as acute outbreaks to long-lasting epidemics (>1 year).
- HEV outbreaks are long-lasting and can affect hundreds to thousands of people.
- Person-to-person transmission is rare and is more frequent in members of the same family who are infected by a common water source.
- ***Age distribution:*** More frequent in adults (3–30%) than in children (0.25–10%).
- ***Sex distribution:*** Men are more commonly infected than women.
- Anicteric and/or subclinical infections may be more frequent in children.
- In 1–4% cases the disease is fulminant.

India

- Approximately 12 million cases occur yearly.
- Out of these 10–40% present with acute hepatitis and 15–45% of these cases go into acute liver failure.
- Superimposed HEV causes 10–15% cases of acute or chronic liver failure in India.

PATHOGENESIS

- Transmission
- Life cycle
- Pathogenicity

Transmission

- Major route is **Fecal-oral** (contaminated water or food).
- Alternative routes are transfusions and vertical transmission.
- These routes are becoming increasingly relevant as more cases are reported each year.
- Blood transfusion has become one of the main transmission routes in developed countries.
- Human-to-human transmission is rare.

Life Cycle

- The life cycle begins with viral entry.
- Hepatitis E virus (HEV) is a quasi-enveloped virus, meaning it can exist in the non-enveloped state (HEV) or can be coated in a lipid-derived membrane [enveloped HEV (eHEV)].
- HEV and eHEV have distinct entry mechanisms.
- Little is known about entry mechanisms for HEV.
- For eHEV, the virus enters the cell through clathrin-dependent and dynamin-dependent, receptor-mediated endocytosis.
- A specific cell surface receptor mediating eHEV entry remains to be identified.
- However, the GTPases Ras-related protein Rab5A (RAB5) and Ras-related protein Rab7A (RAB7) are known to have a role in eHEV entry.
- Upon entering the cell, the envelope of eHEV undergoes lysosome-mediated lipid degradation.
- It uncoats in a poorly understood process to expose the viral mRNA.
- The ORF1 polyprotein (pORF1) containing the RNA-dependent RNA polymerase (RdRp) is translated from the positive-sense (+) strand.
- RdRp then transcribes full-length negative-sense (–) RNA.
- The negative-sense RNA serves as a template for transcribing more full-length positive-sense RNA to be packaged into progeny virions, along with a shorter subgenomic RNA (sgRNA) that contains ORF2 and ORF3.
- The capsid protein (pORF2) and protein ORF3 (pORF3), a viroporin essential for viral release, are translated from the sgRNA.
- pORF3 binds to tumor susceptibility gene 101 protein (TSG101), a member of the endosomal sorting complexes required for transport (ESCRT) pathway.
- The interaction of pORF3 with TSG101 probably promotes budding of progeny virions into multivesicular bodies.
- These then fuse with the plasma membrane to release virions from the cell.
- The lipid envelope of eHEV is thought to be derived from the trans-Golgi network, and viral particles contained in eHEV are associated with pORF3.
- pORF3 has additionally been shown to exhibit viroporin activity.
- It is possible that pORF3 exists in multiple forms to perform distinct functions.
- eHEV released from the apical membrane enters the bile duct, where the lipid envelope is thought to be degraded by detergents and proteases in the bile.
- This would explain why HEV in the feces is non-enveloped.
- On the other hand, eHEV released from the basal membrane of hepatocytes enters the serum in its quasi-enveloped form.
- There it is protected from neutralizing antibodies against pORF2 and pORF3 but is less efficient at infecting cells.

Pathogenicity

- Usually HEV infection presents with an acute self-limiting and asymptomatic presentation.
- However, it varies quite significantly in severity ranging from subclinical to fulminant disease.
- Incubation period can vary from 2 weeks to 2 months (average 40 days).
- Viremia is transient (mainly during the prodromic phase) and disappears at the onset of clinical symptoms.
- Fecal excretion occurs for a few days (5 days average) before jaundice.
- It slows down at the onset of jaundice within 2 to 3 weeks.
- It has a fulminant course in 1–4% (25% in pregnant women).
- Host and viral factors that determine the severity of illness caused by HEV infection are not fully understood.
- The differences in HEV genotypes between different geographical regions might explain the severity of infection during pregnancy.
- Also other host factors like age, contraceptives and pre-existing liver disease may be relevant in certain cases.

MORPHOLOGY

Gross

- Grossly, there are areas of necrosis and collapse of liver lobules.
- These areas appear pale yellow and ill-defined.

Microscopy

- **Acute hepatitis E in the noncirrhotic liver**
  - Lobular disarray.
  - Reticulin framework distortion.
  - Portal tracts: Expanded by a severe mixed polymorph and lymphocytic inflammatory infiltrate.
- **Patients with underlying cirrhosis:**
  - Nonspecific picture
  - May mimic alcoholic hepatitis.

CELL CULTURE SYSTEMS

- The cell culture system of HEV is still limited and relatively ineffective.
- It is confined to HEV 3 and 4.
- The cell culture requires a long incubation period.

CLINICAL PRESENTATION

- High endemic areas
- Low endemic areas
- Extrahepatic manifestations.

High Endemic Areas

- A spectrum of presentations varying from:
  - A mild anicteric systemic illness
  - Typical icteric acute hepatitis
  - Fulminant or acute hepatic failure (ALF).
- Asymptomatic HEV infection appears to be common in these areas.

Anicteric Hepatitis

- It represents a mild liver injury wherein jaundice does not develop.
- It is usually picked up incidentally when abnormal liver function tests (markedly elevated serum transaminases) are detected during the evaluation of minor, non-hepatic systemic symptoms.

Acute Icteric Hepatitis E

- Initially there is a prodrome of non-specific symptoms comprising of malaise, fever, anorexia, nausea and vomiting.
- Specific symptoms include vomiting or right upper abdominal discomfort.
- One week later AST and ALT rise.
- This is followed by heightened urine color and yellow discoloration of eyes.
- The disease course is divided into four phases as follows:
  - *Phase I (viral replication phase):* Patients are asymptomatic during this phase. Laboratory studies demonstrate serological and enzyme markers of hepatitis.
  - *Phase II (prodromal phase):* When seen by a health care provider during this phase, patients are often diagnosed as having gastroenteritis or a viral syndrome.
  - *Phase III (icteric phase):* Patients note dark urine, followed by pale-colored stools.
  - *Phase IV (convalescent phase):* Symptoms and icterus resolve and liver enzymes return to normal.
- Prodromal symptoms and appetite usually improve.
- Jaundice and associated symptoms as well as biochemical abnormalities most often **resolve spontaneously** in 4–6 weeks.

Fulminant or Acute Hepatic Failure (ALF)

- A small proportion (0.5–4%) of HEV-infected persons develop ALF, a **life-threatening form** of acute hepatitis.
- It has the following manifestations:
  - Neuropsychiatric manifestations (hepatic encephalopathy)
  - Abnormal coagulation
  - High mortality
- Occasional patients with acute hepatitis E manifest **intense pruritus,** often with deep jaundice, which persists for several weeks.

Low Endemic Areas

- The disease runs a milder course in these areas.
- Many cases have abnormal liver function tests but no jaundice (Anicteric hepatitis).
- However, certain groups are at higher risk:
  - Elderly men
  - Pre-existing liver-related and other diseases.

- These high-risk groups account for higher mortality in these areas.
- Non-hepatic presentations also appear to be more common in these areas.

Extrahepatic Manifestations

- **GI**: Acute pancreatitis
- **Hematological:** Thrombocytopenia, hemolysis, aplastic anemia
- **Autoimmune:** Membranous glomerulonephritis, Henoch-Schonlein purpura (HSP), arthralgias, skin rash.
- **Neurological:**
  - CNS: Acute transverse myelitis, acute meningoencephalitis, aseptic meningitis, neuralgic amyotrophy, pseudotumor cerebri, bilateral pyramidal syndrome
  - PNS: Guillain-Barré syndrome (GBS), cranial nerve palsy, peripheral neuropathy.

Chronic Infection

- In 2008, a report of a series of patients with prolonged hepatitis E in French solid-organ transplant recipients drew attention to the possibility of chronic HEV infection.
- Chronic HEV infection is defined as the detection of HEV RNA in serum or stool for longer than 6 months.
- Patients who are at risk are those with impaired immune response (solid organ transplant recipients, receiving immunosuppressive drugs, human immunodeficiency virus infection or hematological malignancies).

Superinfection in Chronic Liver Disease

- This is usually seen in hyperendemic areas.
- Liver cirrhosis reduces liver functional capacity.
- Even mild liver injury due to HEV infection may be expected to overwhelm the limited liver reserve, and lead to liver decompensation.
- It begins as an illness resembling typical acute hepatitis.
- However, within a few days, ascites, encephalopathy, or both appear, suggesting decompensated liver disease.
- It is associated with a high mortality.
- The differences between the disease manifestation and other variables between high endemic and low endemic regions are presented in Table 33.1.

Table 33.1: Differences between HEV infection in high endemic and low endemic areas

Features	*High endemic*	*Low endemic*
Geographical distribution	Developing countries	Developed countries
Genotype	1, 2	3, 4
Transmission	Feco-oral (contaminated water)	Contact with animal, consumption of undercooked meat
Reservoir	Human	Animals
Epidemiological pattern	Outbreaks Frequently sporadic	Occasionally sporadic

Contd.

Table 33.1: Differences between HEV infection in high endemic and low endemic areas		
Features	***High endemic***	***Low endemic***
Age	Young adults	Elderly
Characteristic of affected person	Healthy, no prior illness	Co-existent illness often
Clinical presentation	Acute hepatitis, acute liver failure	Acute hepatitis
Severity	Mild to severe	Mild to moderate
Persistent infection	Not reported	In immunosuppressed individuals
Pregnancy association	Associated	Not associated
Extrahepatic manifestations	Pancreatitis more common, others uncommon	Common
Treatment	None required usually	Required

MANAGEMENT

- Diagnosis
- Treatment

Diagnosis

- Routine investigation done is LFT. It may show the following:
  - Increased conjugated bilirubin in 20–30% cases
  - AST is markedly elevated (++++).
  - ALT is markedly elevated (+++)
  - ALP is elevated (+)
  - GGT is elevated (+)
  - Increased unconjugated bilirubin (++)
- Confirmation is done by either of the following:
  - Detection of HEV-specific serum IgM and IgG
  - HEV RNA testing in serum or feces
- Additionally, HEV antigen detection assay may also be done.
- HEV is difficult to culture
- Real-time PCR techniques are more sensitive than virus culture.

Sample collection

- The specimen of choice is **Blood**.
- Venous blood **(3–5 mL)** is to be collected in a sterile dry and labeled vial.
- Hemolysed samples must be avoided as these may interfere with the ability of tests to accurately test the markers.
- To avoid degradation of viral nucleic acid in the specimen, serum should be removed from clotted blood within 4 hours of collection and stored at –20° to –70°C if testing is delayed.
- In case of the outbreak (transmitted by faecal-oral route), in addition to blood samples from patients, water samples and sewage samples may also be collected for RT-PCR.
- Serum samples can be kept at 4–8°C for a maximum of 7 days.

- If required, it is possible to store the serum samples for a longer duration by freezing them at –20°C or lower and transporting to the testing lab on frozen ice packs.
- Sewage and water samples are transported at room temperature.

Serology

- Anti-HEV IgM antibodies are seen in the acute phase which can last for approximately 4 or 5 months.
- IgG starts increasing just after IgM levels rise, i.e. increase is seen from the acute phase until the convalescent phase.
- IgG has been detected up to 4.5 years after the acute phase.
- IgM levels are indicative of acute infection, whereas IgG levels are related to previous contact with HEV.
- In the few last years, some point-of-care tests for the detection of anti-HEV IgM have been developed.
- These kits are based on Gt1 and Gt2 antigens and evaluation has been performed in only a few patients in industrialized areas such as France and Italy.
- Differences in genotype are not considered in the design and selection of antigen proteins.
- Thus, diagnostic kits from different companies yield significant differences in detection, inadequate sensitivity, and high omission factors.
- Several of them have been shown to produce false negative results in patients infected with genotype 1 strains.
- Many diagnostic methodologies are based on HEV genotype-specific antigens, thus limiting the detection of all HEV genotypes.

Molecular testing

- The investigation of choice is RT-PCR.
- A WHO international standard (genotype 3) for nucleic acid testing has been available since 2011.
- Marketed commercial HEV viral load assays appear to have a good sensitivity (20–100 IU/mL) and specificity.
- In immunosuppressed patients since antibodies are not detectable due to delayed or absent production molecular testing becomes very important.
- HEV genotyping is of most importance for research and epidemiologic purposes.
- Conventional RT-PCR products are used for genotyping HEV on different genome segments.
- Viral RNA is detectable in the feces 1 week before the onset of clinical signs to 2 weeks after the onset of clinical signs.
- However, rarely even up to 52 days after the onset of clinical signs viral RNA can be detected.
- In immunocompetent individuals, there is a narrow window (mean 28, range 17–48 days) in which HEV RNA can be detected in serum or feces.
- Therefore, a combination of serology and PCR is required to diagnose HEV infection in such patients.
- The diagnosis of reinfection can be problematic and may be more common than currently appreciated.

- Such patients are typically anti-HEV IgM negative.
- So, the diagnosis depends on the demonstration of HEV RNA by PCR.
- When patients with reinfection present after the viraemic period, they will be PCR and anti-HEV IgM negative but anti-HEV IgG positive.
- The pattern of changes in viral serological markers and viral RNA in acute self-limited infection and chronic HEV infection are shown in Fig. 33.1.

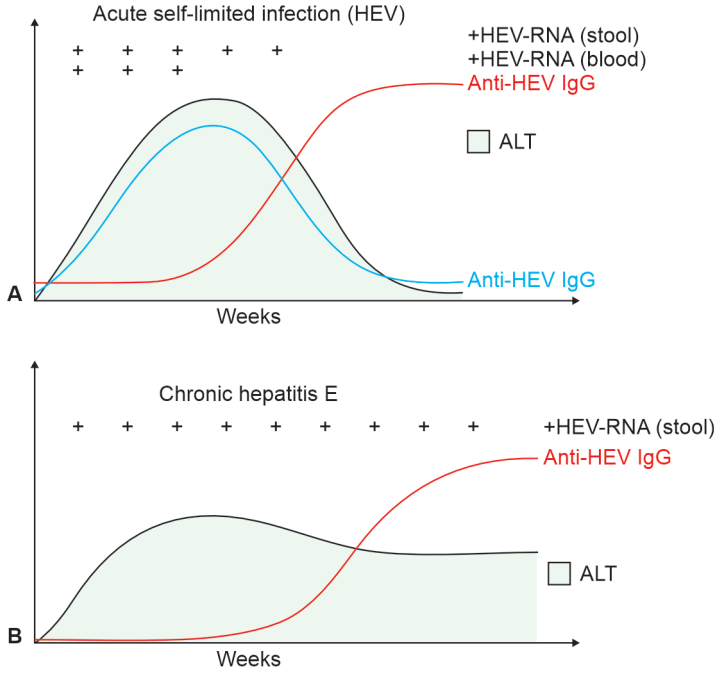

Fig. 33.1: Pattern of changes in viral serological markers and viral RNA in acute self-limited infection and chronic HEV infection

Electron microscopy

- It is useful only in the incubation period and acute phase.
- Usually, patients are asymptomatic in this phase and thus do not come to seek clinical attention.
- Therefore, it is not commonly done.

Treatment

- Acute infections
- Chronic infections
- New treatments

Acute infections

- Most patients recover spontaneously.
- Therefore, only supportive measures aimed at providing symptomatic relief are needed.

- In a few published case reports, ribavirin has been used in patients with severe acute hepatitis E with improvement in laboratory parameters and decline in HEV RNA levels.
- However, in the absence of any controls, it is not possible to ascribe this improvement to the drug, and it remains possible that the recovery was spontaneous.

Chronic infections

- **Restoration of immune status** by stopping or reducing the dose of immunosuppressive therapy or administering antiretroviral therapy in patients with HIV infection.
- Administration of ribavirin or pegylated interferon.
- The former approach is associated with clearance of HEV in about one-third of patients but carries a risk of rejection of the transplanted organ.

Ribavirin

- It appears to inhibit HEV replication by depletion of intracellular guanosine triphosphate pool.
- Other proposed mechanisms:
  - Immune modulation
  - Increased expression of interferon-stimulated genes
  - Viral mutagenesis
  - Inhibition of viral replicative machinery
- Dose: 600–1000 mg/day, for the first three months.

Newer treatments

- Pegylated interferon is limited to a few case reports.
- Though effective in clearing the virus, its administration carries a substantial **risk of rejection of the transplanted organ.**
- Hence, this drug is not a favored option as the first line in patients with solid organ transplantation.
- **Sofosbuvir** is a nucleotide analog developed originally as an inhibitor of RNA polymerase enzyme of hepatitis C virus.
- It has been shown to inhibit replication of genotype 3 HEV in *in vitro* systems based on a subgenomic replicon as well as a full-length infectious clone.
- This inhibition was found to be additive to the effect of ribavirin.

HEV Infection in Pregnancy

- It is a public health problem.
- HEV is responsible for 2400–3000 stillbirths every year.
- The risk increases as pregnancy progresses.
- Viral replication also occurs in the placenta.
- Vertical transmission occurs in 23–50% of cases.
- **Maternal complications:**
  - Fulminant course, resulting in fulminant hepatic failure
  - Post-partum hemorrhage
  - Membrane rupture

- Spontaneous abortions
- Eclampsia
- Stillbirth
- Cerebral edema
- DIC

- **Neonatal complications:**
  - Acquired HEV infection
  - Anicteric or even icteric hepatitis
  - Hypoglycemia
  - Neonatal death.
- The factors that are believed to be responsible for the higher incidence of fulminant disease in pregnancy are as follows:
  - Decreased CD4/CD8 cell count—decrease in cell-mediated immunity.
  - Increased progesterone, estrogen, hCG—suppresses immunity leading to an increase in viral replication.
  - NF-κB downregulation—required for immunity and hepatic regeneration.
  - TGFB, IL4, IL10—decrease in cell-mediated immunity.
  - Decreased glutathione—oxidative stress predisposition.
  - Folic acid deficiency—affects the immune response.
- HEV infection may be a catalyst for coagulopathy, causing a higher probability of postpartum hemorrhage
- Ribavirin with or without pegylated interferon, is specifically contraindicated during pregnancy.
- Main goal is prevention.

VACCINATION

- The following individuals should be vaccinated:
  - At risk of severe disease and/or mortality following HEV infection (e.g. persons with chronic liver disease, pregnant women in disease-endemic areas)
  - Immunosuppressed persons
  - Travelers
  - Outbreaks of hepatitis E in disease-endemic areas
  - Residents of areas where hepatitis E is highly endemic.

First Recombinant Vaccine

- It is a subunit vaccine.
- It contains a 56-kilodalton (KD), truncated (amino acids 112–607) ORF2 protein produced in Spodoptera frugiperda cells.
- Adjuvant used is Alum.
- Tested in a randomized, placebo-controlled, double-blind, phase II trial which enrolled 2000 healthy young soldiers from the Nepalese army showing 95% efficacy.
- **Three, 20 microgram doses** of this vaccine, administered in a **0, 1, 6-month** schedule had a 95.5% protective efficacy during a median follow-up of 804 days.
- Long-term data have not been published and, **unfortunately, this vaccine has not undergone further development.**

Hecolin

- It is also a subunit vaccine.
- HEV 239 (Hecolin®) uses a 26-kilodalton, recombinant, truncated (amino acids 368–606) ORF2 protein expressed in *Escherichia coli*.
- It was tested in a randomized, placebo-controlled, double-blind, phase III field trial in China that included 112,604 healthy men and women aged 18 to 65 years.
- **Three intramuscular doses** of this vaccine (30 μg each) are given at **0, 1 and 6 months**
- It was shown to have 87% efficacy.
- The vaccine protects from genotypes 1 and 4.
- The vaccine requires **further evaluation** in special **risk groups**, such as patients with end-stage liver disease or immunosuppressed individuals.
- Additional studies are also required to determine **what anti-HEV titers can be considered protective** and how **fast** these decrease after vaccination.

PREVENTION AND CONTROL

- Improving overall **hygiene and sanitation**: including proper sewage disposal, and ensuring safe drinking water supplies.
- **Boiling and chlorination** of water inactivate the HEV and may be used during outbreaks of hepatitis E.
- Enhance the **surveillance strategy** in the commercial importation of swine.
- Awareness of raw-undercooked meat.
- Target the preventive measures at persons who have a particularly high risk of developing severe diseases such as pregnant women or persons with pre-existing chronic liver disease.

FUTURE PERSPECTIVES

- More and more animal reservoirs are revealed and we now understand that genotypes 3 and 4 HEV are **zoonotic** and food-borne pathogens.
- Cross-species transmission and host tropism of different **HEV genotypes are still elusive.**
- Recent discoveries about the antigenicity variation between HEV genotypes and enveloped viral particles hidden from neutralizing antibodies suggest new challenges and **questions about the efficacy of the approved vaccine.**
- A HEV-specific treatment approach is needed.
- Development of useful cell culture lines.
- More insights about the basic biology of HEV, such as proteolytic processing of ORF 1 product, functions of the viral proteins, HEV pathogenesis, effective therapeutics are expected to lead to the development of a better vaccine.

POINTS TO REMEMBER

- HEV is becoming increasingly recognized, however, much work remains to be done in understanding its pathogenesis and molecular mechanisms.
- Little is known about key aspects of the viral life cycle.
- A better understanding of these mechanisms could hold the key to developing direct-acting antiviral therapeutics.

- The clinical pathogenesis of hepatitis E disease contains many mysteries as well.
- From the high mortality rate in pregnant women caused by specific strains of the virus to the many extrahepatic symptoms that are being reported in association with infection.
- More clinical data and better cell culture and animal models are needed to understand interactions between the virus and its host.
- More accurate epidemiological data are needed to understand the transmissibility of HEV from foods and to characterize the full extent of the host range of HEV.

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Laboratory Accreditation in India

34

Tanisha Singla

INTRODUCTION

- The most important function of a laboratory is to be recognized as delivering accurate and reproducible results.
- Evaluation or assessment of processes is conducted by a credible and qualified organization.
- Successful completion of this process gives the laboratory recognition that it complies with the quality standards and norms used for the assessment.
- Accreditation is a process by which an authoritative body gives formal recognition that an organization is competent to carry out specific tasks based on third-party assessment and following international standards.

INTERNATIONAL STANDARDS AND STANDARDIZATION BODIES

- A normative document is a document that provides rules, guidelines, or characteristics for activities or their results. It comprises of standards, codes of practice and regulations and technical specifications.
- A standard is a document that provides requirements, specifications, guidelines, or characteristics that can be used consistently to ensure that materials, products, processes and services are fit for their purpose.
- Regulation is any standard that is mandated by a governmental agency or authoritative body.
- Standards may be developed internationally, nationally, or locally.
- Compliance with a standard may be required by the government or another authoritative body, or may be voluntary.
- International standards have the broadest consensus or agreement but may be less specific.
- Standards developed locally have the highest degree of applicability, but may not be useful for comparison with other regions or countries.
- Examples of international organizations are ISO (International Organization for Standardization), CLSI (Clinical and Laboratory Standards Institute), CEN (European Committee for Standardization), WHO (World Health Organization).
- ISO is the world's largest developer and publisher of international standards. 163 countries are members of ISO. It has a Central Secretariat in Geneva, Switzerland that coordinates the system.

- Standards are applicable to many kinds of organizations including clinical and public health laboratories.
- It is a non-governmental organization and forms a bridge between the public and private sectors.
- Many of its member institutes are part of the governmental structure of their countries or have been mandated by the government.
- The work of preparing standards is conducted by ISO technical committees.
- WHO has developed several standards for disease-specific diagnostic laboratories.
- One example is polio, where accreditation is required for a laboratory to participate in the Polio Network for Eradication of Poliomyelitis.

NATIONAL STANDARDS AND TECHNICAL GUIDELINES

- Standards that are developed within a country apply only to national use. They are created by governmental organizations or by recognized bodies with a specific area or domain for application.
- They are developed based on an international standard such as ISO and adapted to the culture and general condition of the country. Examples are:
  - National Accreditation Board for Testing and Calibration Laboratories (NABL), India
  - National Accreditation Body of Indonesia (KAN), Indonesia
  - Irish National Accreditation Board (INAB), Ireland
  - Israel Laboratory Accreditation Authority (ISRAC), Israel
  - National Centre of Accreditation (NCA), Kazakhstan.

When to Apply Standards?

- Standards are used when a laboratory seeks recognition of its ability to use quality practices in carrying out its work.
- Meeting the standards may be a legal requirement, or may be voluntary.
- Three processes may be used to indicate that the laboratory is complying with defined standards—certification, accreditation and licensure.
- **Certification**: The procedure by which an independent body gives *written assurance* that a product, process, or service conforms to specific requirements.
- In this, a laboratory is visited by representatives from a certification body.
- These representatives are looking for evidence of compliance with standards, policies, procedures, requirements, and regulations.
- Primarily, the inspection team checks for the physical presence of texts, procedures and documents.
- **Accreditation:** The procedure by which an authoritative body gives *formal recognition* that a body or person is competent to carry out specific tasks.
- A laboratory is visited by representatives from an accreditation body.
- They look for evidence of compliance with standards, policies, procedures, requirements and regulations. They also observe laboratory staff to ensure that they perform functions and duties correctly and competently.
- Accreditation provides a higher level of assurance because it includes an evaluation of competency.

- **Licensure:** The granting of ability to practise, usually provided by a local governmental agency.
- It is usually based on demonstrated knowledge, training and skills.

Need for Accreditation

- There is acceptance of test results within and beyond borders.
- A customer can easily find an accredited laboratory and there is customer satisfaction along with brand value.
- There is greater control of laboratory process.

Elements of Accreditation

- **Accreditation body:** Oversees the assessments and grants accreditation
- **Standards:** A laboratory must comply in order to gain accreditation.
- **Knowledgeable assessors or inspectors:** To establish compliance with the standards by conducting the assessment.
- **User laboratory:** Required to or voluntarily seeks to comply with the standards by being assessed.

INTRODUCTION TO NABL

- It is an autonomous body founded in 1988. It is a constituent board of Quality Council of India.
- Objective: Provide government, industry associations and industry in general with a scheme of the Conformity Assessment Body's (CAB) accreditation which involves third-party assessment of the technical competence of testing including medical and calibration laboratories, proficiency testing providers and reference material producers.
- The laboratory accreditation services to testing and calibration laboratories are provided by ISO/IEC 17025: **2017**. General Requirements for the Competence in Testing and Calibration Laboratories.
- ISO 15189: **2012** 'Medical laboratories—requirements for quality and competence'.
- The accreditation to proficiency testing providers is based on **ISO**/IEC 17043: **2010** "Conformity assessment—general requirements for proficiency testing".
- For reference material producers it is based on ISO/IEC 17034: **2016** "General requirements for the competence of reference material producers".
- NABL has a mutual relationship with Asia Pacific Laboratory Accreditation Cooperation (APLAC) which in turn has Mutual Recognition Arrangement (MRA) with International Laboratory Accreditation Cooperation (ILAC).
- It has enabled accredited laboratories to achieve a form of international recognition.
- It has reduced the need for *extensive re-testing* of any product or service accepted formally in one economy.

Requirements for Accreditation

- **Create a policy document (quality manual):** It is the greatest document of the quality management system (Level 1) and satisfies every clause mentioned in the standard (ISO 15189). It describes laboratory policies, organization structure, job descriptions and personnel interrelations.

- **Quality manager** is the head of the QMS who reports directly to the lab director and keeps the laboratory audit fit. The manager is also involved in educating and training staff.
- **Establish the Quality Management System (QMS).**
- **Appoint a technical manager** who is usually a senior technical person and is appointed by the Head of the department. He is responsible for the technical workload management and work distribution among existing staff.
- **Process breakdown and quality indicator (QI) identification** quality indicators measure how well an organization meets the needs and requirements of users and the quality of all operational processes. Laboratory process is broken down into its components and micro-components and the common errors are identified.
- **Monitor quality indicators and undertake appropriate corrective action.**
- **Documentation:** Following are the documents of QMS (Fig. 34.1).
- **Indicators for good documentation:**
  - Approved, reviewed and updated regularly.
  - Concise, legible, accurate and traceable.
  - Amendments and revisions are identifiable.
  - Current version is available at points of use.
  - Follows change control procedure.
  - Obsolete documents are separated, identified and retained for a defined amount of time.

Fig. 34.1: Documents of QMS

- Documents can be divided as external documents, internal documents and records.
- **External documents** are maintained by the laboratory for reference purposes and are created by a third party and are formally published for use. They are not subject to the change control procedure of the laboratory, e.g. ISO 15189; 2012 standard, published book.
- **Internal documents:** Documents/reference material created by the laboratory for use within the laboratory. It is subject to the change control procedures created by the laboratory. It is approved by the appropriate person before release for use by the laboratory personnel, e.g. SOP, QM, QSP.
- **Records:** Provide proof/evidence of activity. They have a defined retention period (Table 34.1).

Table 34.1: Retention periods for various records (suggested guidelines for record and specimen retention)

Record/specimen type	*Retention*
Requisitions	2 years
Accession logs	2 years
Maintenance/Instrument logs	2 years
Quality control records	2 years
Blood bank donor/receipt records	10 years

Contd.

Table 34.1: Retention periods for various records (suggested guidelines for record and specimen retention)

Record/specimen type	*Retention*
Blood bank patient records	10 years
Blood bank employee signatures/initials	10 years
Blood bank QC records	5 years
Clinical pathology test records	2 years
Serum/CSF/body fluids	48 hours
Urine	24 hours
Blood/fluid smears	7 days
Microbiology stained slides	7 days
Wet tissue	2 weeks
Surgical pathology (bone marrows) slides	10 years
Paraffin blocks/slides	10 years
Cytology slides	5 years
FNA slides	10 years
Reports (surgical/cytology/nonlorensic)	10 years
Cytogenetic slides	3 years
Cytogenetic reports and images	20 years
Flow cytometry plots/histograms	10 years

OTHER ACTIVITIES

Quality Control

- Here the goal is to identify errors before they impact the real test. It is also called repeatability testing or precision.
- Quality control includes internal quality control (IQC) and external quality assessment (EQA).

Internal Quality Control

- It is based on daily monitoring which includes test procedures performed in the lab using specially prepared materials and repeated measurements on routine specimens and statistical analysis of data.
- **LJ charts:** These were first applied in clinical chemistry by Levey and Jennings as a means to monitor laboratory performance using control materials and are now widely used in hematology for both automated and manual procedures.
- Control material is included in every batch of patients' specimens or at specified intervals during the shift and the result is logged on a control chart.
- It is advisable to use at least one control sample per batch.
- The results obtained with the control samples can be plotted manually on a Levey–Jennings style chart or can be plotted automatically by the instrument.

- When plotting a control chart manually, the mean value and standard deviation (SD) of the control specimen is first established in the laboratory.
- Using arithmetic graph paper, draw a horizontal line to represent the mean (as a base) and, on an appropriate scale of quantity and unit, draw lines representing +2SD and −2SD above and below the mean.
- Plot the results of successive control sample measurements. If the test performance is satisfactory, sequential results oscillate about the mean value and less than 5% of the results fall outside ±2SD.
- Figure 34.2 illustrates a control chart from an automated system. The mean value for each component of the blood count is shown on the left together with the upper (UL) and lower (LL) limits for satisfactory performance, which have been set at +2SD and −2SD respectively.

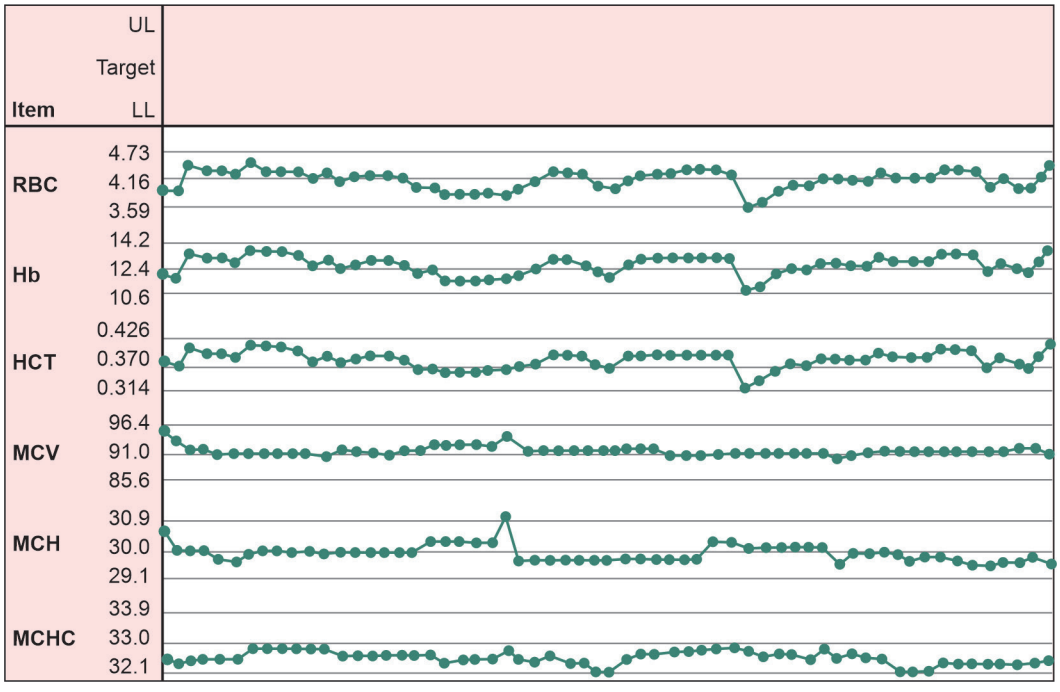

Fig. 34.2: LJ chart from an automated system

Interpretation

- **Satisfactory test:**
  - +2SD to –2SD and <5% of the results fall outside 2SD.
  - 2–3 SD or (–2SD)-(–3SD) (random error): Warning signal
  - <–3SD or >3SD (Blunder or gross error): Action signal
  - Several consecutive similar results on one side of mean: Calibration fault.
  - Consecutive fluctuating values, rising and falling by 2SD: Imprecision.
  - A trend of sequentially increasing or decreasing values with repeated measurements is indicative of drift.
  - One action signal or two warning signal indicate intervention.

- **Duplicate tests:**
  - Duplicate tests on patients' specimens provide another way of checking the precision of routine work.
  - To start the process, test 10 consecutive specimens in duplicate under careful conditions. Calculate the differences between the pairs of results and derive the SD.
  - Subsequent duplicate measurements on any specimen in the same batch of tests should not differ from each other by more than ±2SD. This method will detect random errors but not incorrect calibration.
- **Correlation checks**
  - Any unexpected result in a test is checked to see if it can be explained in a clinical grounds or whether it correlates with other tests.
  - For example, a low MCHC may be confirmed by demonstrating hypochromic red cells on a stained blood film; a high MCV must correlate with macrocytosis.

External Quality Assessment

- Also known as proficiency testing.
- It is defined as a system whereby a set of reagents and techniques are assessed by an external source and the results of the testing laboratory are compared with those of an approved reference laboratory or agency.
- EQA complements IQC, providing a long-term, retrospective assessment of participant performance against that of other laboratories and is a requirement for medical laboratories for accreditation to ISO15189.
- The operation of EQA schemes is based on the circulation of survey material to a number of participating laboratories or individuals who return their results to the EQA organizer for analysis.
- Performance of each participant is evaluated against the target value for the material and out of consensus results identified. Appropriate action is taken in response to unsatisfactory performance.

PROCESS OF ACCREDITATION

- **Application for accreditation of laboratory:** The laboratory is required to apply in the prescribed application form along with a quality manual that should describe the management system in accordance with ISO/ IEC 17025: 2005 or ISO 15189: 2012 or ISO/IEC 17043:2010 or ISO 17034: 2016 whichever is applicable.
- **Acknowledgment and scrutiny by NABL secretariat:** NABL Secretariat on receipt of application form, the quality manual and the fees issues an acknowledgment to the laboratory indicating a unique ID number, which is used for correspondence with the CAB.
- **Pre-assessment of the laboratory by the lead assessor:** In case there are no inadequacies in the quality manual a pre-assessment visit of the laboratory is organized by the lead assessor appointed by NABL. The pre-assessment of the laboratory is conducted to evaluate non-conformities (if any) in the implementation of the quality system. The lead assessor submits a pre-assessment report to the NABL Secretariat with a copy to the laboratory. The Laboratory takes corrective actions on the non-conformities and submits a report to the NABL Secretariat.

- **Final assessment of the laboratory by the assessment team:** After the Laboratory has taken satisfactory corrective actions, NABL finalizes the constitution of assessment team in consultation with the laboratory. The team includes the lead assessor and technical assessors. The laboratory's technical competence to perform specific tasks is evaluated. The non-conformities if identified are reported in the assessment report. It also provides a recommendation for a grant of accreditation or otherwise. The report prepared by the assessment team is sent to the NABL Secretariat.
- **Scrutiny of assessment report by NABL Secretariat:** The assessment report is examined by the NABL Secretariat and follow-up action as required is initiated. Laboratory has to take necessary corrective action on non-conformities and submit a report to the NABL Secretariat within 60 days.
- **Recommendation for accreditation by accreditation committee:** After satisfactory corrective action by the laboratory, the accreditation committee examines the assessment report and makes appropriate recommendations regarding accreditation of the laboratory to the chairman, NABL.
- **Approval for accreditation by Chairman, NABL.**
- **Issue of NABL certificate by NABL:** When the recommendation results in the grant of accreditation, NABL issues an accreditation certificate which has a unique number and NABL hologram, discipline, date of validity along with the scope of accreditation.
- The NABL accreditation certificate is valid for 2 years.
- NABL conducts annual surveillance of the laboratory at intervals of one year which is aimed at evaluating continued compliance to the requirements of ISO/ IEC 17025: 2005 or ISO 15189: 2012 or ISO/IEC 17043:2010 or ISO 17034: 2016 whichever is applicable and relevant specific criteria and NABL Policies.
- The accredited laboratory is subjected to re-assessment every 2 years. It has to apply 6 months before the expiry of accreditation to allow NABL to organize an assessment of the laboratory, so that the continuity of the accreditation status is maintained.

NABL vision: To be the world's leading accreditation body and to enhance stakeholders' confidence in its services.

TAKE HOME MESSAGE

- Accreditation is a positive tool for lab improvement.
- A continuous process of improvement of the quality management system.
- It helps in providing an efficient health care system.
- It is an accomplishment to be accredited but it is an achievement to maintain accreditation.

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35 Molecular Characterization of Circulating Tumor Cells

Poonam Yadav

INTRODUCTION

- Circulating tumor cells (CTCs) are defined as neoplastic cells originating from either primary or metastatic tumors and circulating freely in the peripheral blood of cancer patients.
- Cancer cells from almost any kind of malignant tumor can, and often do, enter into the bloodstream.
- They are then transported to distant organs where they may lodge and form metastases.
- Death from cancer in these patients is the result of metastatic dissemination.

HISTORICAL OVERVIEW

- They were first described ***in 1869 by Dr Thomas Ashworth,*** an Australian physician.
- When observing under the microscope the blood of a man with metastatic cancer, he **discovered "cells identical with those of cancer itself".**
- He postulated that they **"may tend to throw some light upon the mode of origin of multiple tumors existing in the same person".**
- In 1934, Pool and Dunlop did the first deliberate search for cancer cells in the blood of living patients.
- Their technique was crude and consisted of the following steps:
  1. 5 mL of oxalated blood was hemolyzed with 15% acetic acid and the centrifuged sediment was fixed with 10% formalin in alcohol, then embedded in paraffin and step-sectioned.
  2. "Large spherical cells with hyperchromatic nuclei" were seen in 17 of 40 cancer cases.
  3. One patient with a benign gastric ulcer also showed these cells.
- The number of cancer cells within the bloodstream (excluding leukemias) is infinitesimal when compared with the number of normal blood cells.
- Thus, it is extraordinary to find cancer cells in routine blood smears, though such cases have been reported called "**carcinocythemia".**
- A systematic search for cancer cells in the circulating blood of cancer patients became a major research target in the 1950s and 1960s.

- It was hoped that it would serve as a possibly valuable predictor of disease progression and guide to therapy.
- The task of separating or concentrating these cells and giving them their proper identification proved to be a major technical problem.
- Therefore, interest in the research community waned over the next few years.

Early Technical Methods

- The great variety of techniques that had been proposed for processing peripheral blood specimens is a reflection of dissatisfaction with any one of them.
- Separation of the red cell mass was accomplished initially by ***hemolysis***. Following agents were employed:
  - Water (Quensel, 1921),
  - Acetic acid (Pool and Dunlop, 1934; Fisher and Fisher, 1959; Romsdahl et al, 1965), or
  - Saponin (Engell, 1955; Nedelkoff et al, 1961; Ericksson, 1962).
- ***Enzymes***, such as streptolysin-O, were used by Potter and Malmgren (1958).
- ***Sedimentation***, usually with an accelerating factor such as:
  1. Fibrinogen (Buckley et al, 1950; Sandberg and Moore, 1957; Roberts et al, 1958),
  2. Dextran (Alexander and Spriggs, 1960; deCarvalho, 1960; de Mello, 1963),
  3. Phytohemagglutinin (Li and Osgood, 1949), or
  4. Hemolymph heteroagglutinin (Watne et al, 1966), were also tried.
- Separation of cell types may be facilitated by differential sedimentation or centrifugation after suspending the blood in a solution of:
  1. Albumin (Fawcett et al, 1950; McGrew, 1954; Roberts et al, 1958),
  2. Gum acacia (Spear, 1948), or
  3. Silicone of specific gravity adjusted between that of the heavier red blood cells (1.092–1.097) and the lighter cancer cells and lymphocytes (1.056–1.065) (Seal, 1959).
- Danielsson (1961) used **a spiral centrifuge** for separation of the lighter cellular elements.
- However, it offered little practical advantage for the relatively small volumes of blood is usually examined.
- Differential centrifugation was also used by some workers.
- However, none of these methods worked perfectly.
- After processing the blood by any one, or several, of these methods, the final cell suspension was transferred to either of the following:
  1. Membrane filters (Seal, 1956) or
  2. Centrifuged onto glass slides (Ericksson, 1962), or
  3. Simply smeared on glass slides and stained.
- **Membrane filters** proved to be more convenient and popular and had the advantage of possibly better quantitation.
- The choice of stain depended on personal preference.
- Hematoxylin and eosin or the Papanicolaou stain were commonly used with all types of preparations.
- Wright-Giemsa or other hematologic stains, and the metachromatic fluorochrome acridine orange (de Mello, 1963) were generally restricted to smears on glass slides.

DETECTION METHODS

- Due to the rare nature of CTCs, the process typically requires both enrichment and detection steps.
- For enrichment, four approaches currently exist:
  1. Size-based
  2. Density-based
  3. Immunomagnetic separation
  4. Microfluidic-based.
- In ***size-based enrichment techniques***, diluted whole blood is passed through a filtration devise with specific-sized pores.
- CTCs are captured based on differences in cell size between CTCs and white blood cells.
- ***Density-based enrichment*** utilizes Ficoll (or a similar density gradient medium) to enrich for mononuclear cells (including CTCs) from other blood components.
- ***Immunomagnetic separation*** involves the use of iron-conjugated antibodies targeted toward CTCs (e.g., EpCAM; positive selection) or contaminating blood cells (e.g., CD45; negative selection) and incubation in a magnetic field.
- In ***microfluidic-based techniques***, whole blood is slowly passed across a chip-based surface and isolated using CTC-targeted antibody-coated microposts (CTC Chip and iChip).

CONTEMPORARY METHODS OF IDENTIFICATION OF CIRCULATING CANCER CELLS

- Broadly these methods can be divided into two categories as follows:
  1. **Protein-Based**
     - Immunocytochemistry
     - Immunofluorescence
     - Flow Cytometry
  2. **Nucleic Acid-Based**
     - Fluorescence *in situ* hybridization (FISH)
     - Reverse transcription-polymerase chain reaction (RT-PCR) and Reverse Transcription Quantitative PCR (RT-qPCR)
     - Microarrays
     - Sequencing

Protein-based Methods

Immunocytochemistry

- Differences in antigen expression could be a useful parameter for recognizing circulating cancer cells.
- *Epithelial cytokeratins* are the most commonly used tissue-specific markers.
- They constitute a major constituent of carcinomas and are not found in the hemopoietic cells of blood or marrow.
- Other markers used in several studies include:
  1. Prostate-specific antigen (PSA), which is found in cells of prostatic epithelial origin.
  2. Tyrosinase, which is expressed in melanoma.
  3. CEA and mucin antigens, observed in adenocarcinomas.

- The detection of tumor cells occurs after enrichment by density gradient or immunomagnetic separation.
- Maximum sensitivity reported is one tumor cell among 10^5 to 10^6 nucleated cells in blood or marrow.

Double Labeling

- It is done by labeling breast cancer cells with both EMA (epithelial membrane antigen) and an antibody to estrogen receptor (ER).
- Thus, it is possible to distinguish ER-positive and ER-negative tumor cells.
- The possibility of identifying and characterizing circulating cancer cells immunocytochemically in ways that might predict their potential to form metastases and/or respond to therapies has been suggested.

Immunofluorescence

- It is the primary means by which CTCs have been interrogated at the protein level, using specifically targeted antibodies.
- CTC enrichment techniques that have been employed prior to immunofluorescent staining include:
  1. Immunomagnetic approaches (both positive and negative selection)
  2. Density gradient centrifugation
  3. Microfluidic chip-based approaches
- Using immunofluorescence, CTCs have been characterized for expression of many markers including:
  1. HER2
  2. EGFR (epidermal growth factor receptor)
  3. Androgen receptor
  4. Prostate-specific antigen (PSA)
  5. Estrogen receptor
  6. Progesterone receptor.
- Immunofluorescence has the following advantages:
  - The ability to examine the presence or absence of expression, as well as protein localization and co-localization with additional proteins.
  - The ability to examine many proteins of interest simultaneously, limited only by the filter capacity of the investigator's microscope.
  - Visually confirm that expression is in CTCs and not contaminating cells.
  - Visualize variations in protein expression levels (it is important to note that this may also be seen as a disadvantage if not properly standardized).
- However, it also has certain limitations as follows:
  1. Primary limitation is that the data obtained using this approach is largely qualitative.
  2. Limitations in assay sensitivity (i.e., enough antigens need to be present to display a visible signal).
  3. Bleed-through from additional fluorescence channels can interpret results confusing.

4. Using this approach, result interpretation can be more difficult to standardize (i.e., what constitutes a true positive or negative signal), although automated CTC analysis approaches are evolving to help address this issue.

- The biggest advantage is the ability to identify the presence or absence of a particular therapeutic target molecules.
- This expands the availability of targeted therapies to patients who would previously be considered ineligible based on the characteristics of their primary tumor.
- For example, a breast cancer patient with HER2 negative primary tumor is an excellent example of a setting in which CTC characterization could augment patient care.
- The detection of HER2 positive CTCs in a patient with a HER2 negative primary tumor could predict response to HER2 targeting agents.
- This would increase the availability of these personalized treatment options to a wider group of patients.

Flow Cytometry

- Primary limitation of immunofluorescence is that the data is largely qualitative.
- Quantitative analysis of these rare cells may be advantageous.
- Flow cytometry offers the ability to easily sort and collect characterized CTCs using fluorescence-activated cell sorting (FACS) technology.
- It has the following advantages:
  1. Examination of not only the presence or absence of marker expression but the level of expression can be measured in a quantifiable fashion.
  2. Easily perform multi-marker analysis on a single sample, limited only by laser and fluorescent filter set availability.
  3. Ease of sorted sample collection and downstream characterization using other approaches.
- Following are the disadvantages:
  1. Limitations exist with regard to assay sensitivity even when combined with pre-enrichment steps.
  2. Inability to visually confirm that results are from CTCs and not due to leukocyte contamination.
- The use of flow cytometry for CTC characterization in the clinic could provide similar benefits as those recognized for immunofluorescent techniques.

Nucleic Acid-Based CTC Characterization Approaches

Fluorescence In Situ Hybridization (FISH)

- At the genomic level, FISH has been utilized to interrogate CTCs for the following changes in individual genes:
  1. Gene copy number
  2. Gene rearrangement
  3. Gene deletion
  4. Chromosomal changes, such as select arm deletion or amplification.
- Before FISH analysis, enrichment is performed via any of the previously mentioned methods.

- Following enrichment, isolated CTCs from metastatic breast, prostate, and lung cancers have been examined.
- The examination is done either on-platform or after being cytospun onto charged glass slides.
- Several common genomic aberrations and amplifications have been studied as given below:
  1. HER2
  2. Anaplastic lymphoma kinase (ALK)
  3. Phosphatase and tensin homolog (PTEN)
  4. Androgen receptor
  5. EGFR and
  6. TMPRSS2:ERG (transmembrane protease serine 2:ETS-related gene) fusions.
- The advantages of FISH are as follows:
  1. Assessment of the genomic characteristics of individual CTCs with visual confirmation is done.
  2. Easily defined cut-off/threshold values based on quantifiable ratios of mutation to parent chromosome can be assigned.
  3. Automated FISH enumeration systems are available.
- FISH has the following limitations:
  1. It interrogates CTCs at the genomic level and therefore results may not truly reflect CTC phenotype at the functional protein level.
  2. FISH assessment does not provide information regarding markers whose regulation and/or function rely on epigenetic changes, phosphorylation, or appropriate protein localization.

Reverse Transcriptase: Polymerase Chain Reaction (RT-PCR)

- With regards to CTC analysis, RT-PCR has been utilized to achieve the following:
  1. Detect the presence or absence of CTCs
  2. Specific molecular characterization
- The target transcripts utilized for CTC detection are predominantly of either epithelial- or tissue-specific origin like EpCAM, prostate-specific membrane antigen [PSMA], mucin-1 [MUC-1], etc.
- It is presumed that these genes are not transcribed by contaminating leukocytes.
- RT-PCR also requires prior enrichment for CTCs.
- Limitations of RT-PCR are as follows:
  1. Assay sensitivity is high. However, specificity is lower as a result of illegitimate transcription and false positives. To overcome this limitation many workers have chosen to utilize RT-qPCR.
  2. The major advantage that RT-qPCR has over RT-PCR is the ability to set defined cut-offs, in the form of Cq values.
  3. Thus, false positives are reduced based on levels of illegitimate transcription observed in healthy donor blood samples.
  4. The inability to visually confirm that signals obtained are from CTCs and not due to leukocyte contamination.
  5. Analysis of single CTCs is still technologically challenging using this approach with few studies having published results from patient samples.

Microarrays

- The following types of microarrays have been utilized for CTC detection:
  - **Gene expression arrays** that provide information about samples at the RNA level, in particular the up/down-regulation of suspected and novel transcripts.
  - **Comparative genomic hybridization arrays** which provide information about samples at the DNA level, including copy number variations, specific mutational variants, or global genomic changes.
- Enrichment is done mostly by either Immunomagnetic enrichment or density gradient centrifugation.
- Microarray has certain advantages:
  1. It provides an automated analysis.
  2. Pre-determined cut-off values can be set, thereby standardizing interpretation.
  3. Direct comparison of a multitude of disease settings (e.g., CTCs to primary/metastatic tumors, CTCs in treatment responders versus non-responders, CTCs at baseline versus following systemic treatment, etc.)
  4. Potential for novel biomarker identification and/or CTC gene signatures is there.
- However, it has the following limitations:
  1. The necessity for specialized bioinformatics personnel for the analysis and interpretation of the massive amount of data that can be generated using this approach.
  2. Individually identified genes (gene expression arrays) have to be validated using RT-qPCR.
  3. It is costly.
  4. It is difficult to assess sample purity to determine if results are from CTCs or contaminating leukocytes.
  5. Limitations with regard to sensitivity that can make single-cell analysis difficult.
  6. There is a paucity of data as there are only a few studies reporting on arrays using individual CTCs.

Sequencing

- Sequencing is an umbrella term that encompasses several different methodologies.
- Broadly they can be of two types:
  1. **Traditional gene sequencing approaches**
     - Sanger sequencing
     - Pyrosequencing
     - MALDI-TOF sequencing
     - Targeted sequencing approaches such as allele-specific RT-PCR and qPCR melting curve analysis.
  2. **Next-generation sequencing platforms**
     - Roche 454™ pyrosequencing
     - Life Technologies SOLiD™ sequencing
     - Ion Torrent™ sequencing
     - Illumina HiSeq™
     - Helicos HeliScope™
     - Pacific Biosciences PacBioRS™.

- Sequencing technology is a powerful tool for the analysis of specific genomic aberrations.
- This is especially true in the setting of cancer.
- The technique can be applied to both genomic DNA and transcribed RNA sequences in the form of cDNA.
- With regards to CTC analysis, sequencing tends to be applied more frequently at the level of RNA.
- Total RNA or mRNA is extracted from CTCs following enrichment using either of the following methods:
  - Immunomagnetic methods.
  - Density gradient centrifugation.
  - Microfluidic chip-based approaches.
- Isolated RNA is reverse transcribed into cDNA.
- It is PCR amplified using primers that are specific to the mutant/target region.
- Amplified mutations can be detected using **either gel electrophoresis** for known length transcripts, and/or analyzed with one of the several **commercially available sequencing platforms.**
- Sequencing technique has the following advantages in CTC detection:
  - Single nucleotide alterations can be identified, since minor aberrations can result in significant phenotypic changes and may be important for predicting response to selected therapies.
  - Results from sequencing are presented as either positive or negative and do not appear as gradations as with immunofluorescence.
  - Analysis can be automated to reduce interpreter bias.
- There are certain limitations which are as follows:
  - Limitations with regard to sensitivity that make single-cell analysis difficult. There is a need for a minimum of 50 or more CTCs for adequate results.
  - Leukocyte contamination and the inability to visually confirm the source of amplified transcripts can lead to false positive/negative results.

APPLICATIONS OF CTC DETECTION

- **Monitoring treatment:** CTC detection is valuable in monitoring response to treatment.
- **Targeted therapies:** Detection of CTCs and characterization of molecular abnormalities help in delivering targeted therapies.
- **Early detection:** It is useful in the early detection of cancer even before it becomes clinically apparent.
- **Biology of metastasis:** A better understanding of CTCs will lead to further unraveling the mysteries of tumor metastasis.
- **Biomarker discovery:** Sequencing techniques will likely throw up newer molecular abnormalities. This will lead to the discovery of newer biomarkers of cancer.

CURRENT LIMITATIONS AND FUTURE DIRECTIONS

- Despite the development of numerous CTC platforms using various combinations of enrichment and detection steps, capture of these cells is still technologically challenging.

- This is due to the following reasons:
  - Their rare nature (~1 CTC per 10^5–10^8 white blood cells).
  - The potential presence of contaminating cells can lead to false positive identification (i.e., non-tumor epithelial cells, circulating endothelial cells).
  - The lack of a globally accepted marker for capture of all CTCs (i.e., some CTCs may lose EpCAM (epithelial cell adhesion molecule)/CK (cytokeratin) expression as they enter the bloodstream via a process known as epithelial-to-mesenchymal transition.
  - The most prominent limitation that currently exists in the molecular characterization of CTCs is the low number of CTCs collected, especially from those patients with early-stage disease.
  - These cells are very delicate in nature and can be easily lost.
  - They tend to be quite heterogeneous, therefore it is difficult to draw conclusions about treatment if only a small population of cells are available for analysis.

POINTS TO REMEMBER

- Tumor cells continuously change their genetic and molecular makeup over time and under the selection pressure of treatment.
- To reduce cancer mortality, it is essential to timely diagnose the disease before metastasis progresses beyond control.
- Also, drug resistance should be avoided by effective and personalized therapy.
- Analysis of CTCs might allow clinicians and researchers to achieve such personalized and effective strategies.
- They are sensitive biomarkers that allow early diagnosis, real-time monitoring, and molecular characterization.
- However, the current state of CTC molecular characterization is truly in its infancy.
- The potential of CTC detection is restricted by limitations in detection and isolation methods.
- It is extremely challenging to establish a highly sensitive and specific method that can capture the full spectrum of CTCs.
- In addition, different methods of CTC isolation may produce different results.
- Therefore, standardized assay protocols for sample preparation, handling, enrichment, detection, and evaluation are of utmost importance.
- Despite all these challenges rapid progress is being made.
- In the future, the clinical application of CTC molecular characterization will greatly benefit patients.

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Section

6

Histopathology

Approach to Vesiculobullous Disorders of Skin

36

Anisha Manocha

INTRODUCTION

- Vesiculobullous disorders comprise a large group of clinically polymorphic diseases.
- Historically, pemphigus and pemphigoid were broad terms used in their clinical description.
- With advancements in knowledge we have acquired a better understanding of the broad variety of these diseases and the specific histopathologic mechanism of each.
- The diagnosis is often dependent on the skin biopsy and careful histopathologic evaluation.
- Determining the exact location of the blister in relationship to the epidermis is very important.
- While approaching blistering diseases, there are three fundamental things to consider:
  1. Site or level of the blister (or the lowest level of vesiculation): Subcorneal, midepidermis, suprabasal, subepidermal.
  2. Findings that implicate the mechanism of blister formation (spongiosis, acantholysis, blistering degeneration, or epidermolysis).
  3. The type of inflammation (neutrophilic, lymphocytic, eosinophilic, mixed), if present.
- A ''top-down'' approach [evaluating the histopathologic specimen beginning at the stratum corneum and moving inferiorly through the epidermis to the basement membrane zone (BMZ) and then the dermis] is recommended.
- However, it is important to note that the histopathologic diagnosis must be considered in the context of the clinical stage of the disease, the stage of the blister, and the location of biopsy (perilesional, lesional, or nonlesional).

How to BIOPSY a Vesiculobullous Eruption?

- **Avoid the ulcerated portions:** This does not show the presence of a roof in the biopsy.
- Also, secondary changes such as inflammation and necrosis may mask the relevant histological findings.
- **Choice of lesion:** Fresh (less than 24–48 hours old), intact, and non-excoriated bullae, with normal or erythematous perilesional skin should be sampled.
- The patient should not be on steroids till a month prior to biopsy to prevent false-negative results.
- A 4 mm punch biopsy from perilesional skin or mucosa, i.e. within a 1 cm radius from a blister or erosion is recommended for DIF.

- These biopsies should not be dissected during grossing.
- The biopsy for histopathology is transported in 10% neutral buffered formalin, whereas the biopsy for Direct immunofluorescence (DIF) is transported in Michel's medium.
- Patient's serum or blood should be used for Indirect IF.

Alternative Biopsy Approaches

- A small (1–2 mm) blister is chosen and removed in entirety with an 8-mm punch with the blister at the center of the punch.
- It is then bisected and submitted half for H&E and half for DIF.
- In case of a large blister the edge is biopsied using a 6 or 8 mm punch.
- Biopsy is done after using a marker to delineate the line along which the specimen is bisected to provide half for H&E and half for DIF.
- In case of a large flaccid blister the third approach is followed by taking 2 biopsies.
- The first biopsy is taken at the edge of the blister, including approximately 10% blister and 90% perilesional skin.
- The specimen is bisected at the bedside to demonstrate the ''take-off'' point of the blister for H&E.
- The second biopsy is taken from perilesional skin (1 cm from lesional skin) and is submitted for DIF.

IMMUNOLOGY

- These vesiculobullous disorders are autoimmune disorders in which the autoantibodies target various antigens located in the epidermis.
- These are summarized in Table 36.1.

HISTOPATHOLOGY

- Based on the histopathological approach vesiculobullous lesions are further classified as summarized in Table 36.2.
- The type of inflammatory infiltrate is also quite characteristic as presented in Table 36.3.

Table 36.1: Antigens targeted by autoantibodies in vesiculobullous disorders

Vesiculobullous disorder	*Antigen*
Pemphigus vulgaris	Desmoglein 1 and 3
Paraneoplastic pemphigus	Desmoglein 1 and 3, plakins
Pemphigus foliaceous	Desmoglein 1
IgA pemphigus	Desmoglein 3, Desmocolin 1 and 2
Pemphigus herpetiformis	Desmoglein 1
Cicatricial pemphigoid	BP 180, laminin V
Bullous pemphigoid	BP180 and 230
Epidermolysis bullosa acquisita	Type VII collagen
Epidermolysis bullosa simplex	Keratin 5 and 14
Epidermolysis bullosa junctional	Laminin V and type XVII collagen
Epidermolysis bullosa dystrophic	Type VII collagen
Erythema multiforme	Desmoplakins
Dermatitis herpetiformis	Tissue transglutaminase

Table 36.2: Major blistering disorders based on the histopathological approach

Subcorneal bulla	
Subcorneal bullae without acantholysis	***Subcorneal bullae with acantholysis***
⊥ Subcorneal pustular dermatosis ⊥ Acute generalized exanthematous pustulosis	⊥ Pemphigus foliaceous ⊥ Bullous impetigo ⊥ Staphylococcal scalded skin syndrome
Intraepidermal bulla	
Intraepidermal bulla without acantholysis	***Intraepidermal bulla with acantholysis***
⊥ Allergic contact dermatitis ⊥ Id reaction	⊥ Pemphigus group of blistering diseases ⊥ Hailey-Hailey disease (benign familial pemphigus) ⊥ Keratosis follicularis (Darier disease) ⊥ Transient acantholytic dermatosis (Grover disease) ⊥ Herpes simplex and herpes zoster infection
Subepidermal bulla	
Subepidermal bullae without inflammation	***Subepidermal bullae with inflammation***
⊥ Epidermolysis bullosa ⊥ Porphyria cutanea tarda	⊥ Bullous pemphigoid, cicatricial pemphigoid, Pemphigoid gestationis ⊥ Dermatitis herpetiformis ⊥ Linear IgA bullous disorder ⊥ Epidermolysis bullosa acquisita

Table 36.3: Inflammatory infiltrate in major vesiculobullous disorders

Inflammatory infiltrate	*Vesiculobullous disorder*
⊥ Lymphocytes	⊥ Bullous erythema multiforme ⊥ Paraneoplastic pemphigus ⊥ Allergic contact dermatitis
⊥ Neutrophils	⊥ Linear IgA dermatosis ⊥ Bullous Lupus erythematosus ⊥ Dermatitis herpetiformis
⊥ Eosinophils	⊥ Bullous Pemphigoid ⊥ Arthropod bite reaction
⊥ Neutrophils/Eosinophils/Mixed inflammation	⊥ Pemphigus vulgaris ⊥ Dermatophyte infection

SUBCORNEAL BULLAE WITHOUT ACANTHOLYSIS

I. Subcorneal Pustular Dermatosis (Sneddon-Wilkinson Disease)

- **Clinical features**
  - Large flaccid pustules on the trunk and body folds in middle-aged females
  - Pustules are arranged in a serpiginous or annular pattern.
  - May be associated with monoclonal gammopathy, especially IgA paraproteinemia.
- **Microscopy**
  - Large unilocular subcorneal aggregate of neutrophils.
  - Adjacent epidermis shows psoriasiform acanthosis with the presence of neutrophils between the keratinocytes.

- Superficial mixed dermal inflammatory infiltrate composed primarily of neutrophils and lymphocytes is seen.

- **Immunofluorescence:** Negative for IgG, IgA, IgM and C3.

II. Acute Generalized Exanthematous Pustulosis

- **Clinical features:**
  - Acute onset of sterile pustules, usually within hours of starting medicines such as beta-lactam and macrolide antibiotics
  - Subsides after stopping the associated medication.
- **Histopathology:**
  - Subcorneal/Intraepidermal pustules with spongiosis of the epidermis
  - Dermis shows a mixed inflammatory infiltrate with eosinophils and edema of the papillary dermis.
- **Immunofluorescence:** Negative for IgG, IgA, IgM and C3
  - Differential diagnosis: Scabies
  - Also shows subcorneal neutrophils and dermal eosinophils.
  - Presence of the mite and remnants help to differentiate.

SUBCORNEAL BULLAE WITH ACANTHOLYSIS

I. Pemphigus Foliaceous

- **Clinical features**
  - Found primarily in middle-aged population.
  - Crusts and shallow erosions on healthy-appearing skin.
  - Trunk is a common site.
  - Mucous membrane involvement is rare, in contrast to Pemphigus vulgaris where it is common.
  - *Pemphigus erythematosus:* Variant of Pemphigus foliaceous which is associated with Lupus erythematosus.
- **Histopathology**
  - Subcorneal bulla with acantholysis.
  - Acantholytic cells were seen floating in bulla cavity lumen.
  - Often seen clinging to stratum corneum (cling-on).
- **Immunofluorescence:** Characteristic chicken wire pattern of IgG deposits in the upper epidermis. Negative for IgA, IgM and C3

II. Bullous Impetigo

- **Clinical features:**
  - Found primarily in children.
  - Confluent pustules and honey-colored crusts.
  - Associated with a staphylococcal or Group A Streptococcal infection.
- **Histopathology:**
  - Subcorneal bulla containing neutrophils.
  - The roof of bulla shows parakeratosis.
  - The floor of bulla contains acantholytic cells.
  - Demonstration of bacteria by Gram stain or culture is diagnostic.
- **Immunofluorescence:** Negative for IgG, IgA, IgM and C3

III. Staphylococcal Scalded Skin Syndrome

- **Clinical features:**
  - Affects infants and young children.
  - Diffuse erythema and superficial epidermal desquamation.
  - Generally, a concurrent staphylococcal infection is present (Staphylococcal exfoliative toxins A and B target Desmoglein 1).
- **Histopathology:**
  - Subcorneal or intragranular bulla with focal acantholysis.
  - Inflammatory infiltrate is scant but predominantly neutrophilic.

INTRAEPIDERMAL BULLAE WITH ACANTHOLYSIS

Pemphigus

- It is a group of autoimmune blistering disorders.
- Depending on the plane of separation:
- Superficial type: Pemphigus foliaceus (mentioned previously).
- Deep type: Pemphigus vulgaris and pemphigus vegetans.

I. Pemphigus Vulgaris

- **Clinical features:**
  - Numerous flaccid bullae on the trunk and scalp.
  - Oral mucosal involvement is frequently present and may at times be the presenting feature.
  - *Pemphigus vegetans:* Verrucous plaques in flexural and intertriginous zones. Oral involvement is seen.
  - *Paraneoplastic pemphigus:* Oral and cutaneous bullae/ erosions in patients with an underlying malignancy which is usually a hematolymphoid malignancy.
- **Histopathology:**
  - ***Pemphigus vulgaris:***
    - Suprabasal bulla with associated rounded acantholysis (Fig. 36.1).
    - The basal keratinocytes remain attached to the basement membrane forming a "Row of tombstones appearance".
    - The acantholysis also extends into the hair follicle epithelium which is very characteristic of pemphigus vulgaris.
    - Eosinophilic spongiosis is seen in the early stages.
    - Dermis shows a mixed inflammatory infiltrate.
  - ***Pemphigus vegetans:***
    - Marked epidermal acanthosis and hyperplasia with intraepidermal eosinophilic microabscesses.
    - Acantholysis may not be seen.

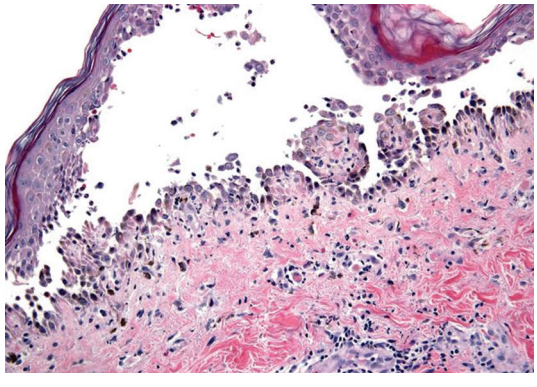

Fig. 36.1: Pemphigus vulgaris showing suprabasal acantholysis with a row of tombstones appearance

- ***Paraneoplastic pemphigus:***
  - A combination of suprabasal acantholysis with interface changes in other areas is a characteristic feature.

- **Direct immunofluorescence:**
  - Biopsy for DIF should be taken from the perilesional skin.
  - IgG and/or C3 show positivity in the intercellular spaces, around the keratinocytes giving a "Fishnet appearance" (Fig. 36.2).
  - Paraneoplastic pemphigus: In addition to the intercellular pattern, granular deposits of C3 are seen at the dermoepidermal junction.
- **Indirect immunofluorescence:**
  - Serum examination can also be performed to detect specific patterns of immunoglobulin deposition on various tissue substrates
  - Autoantibodies to desmoglein 1: Pemphigus foliaceous
  - Autoantibodies to desmoglein 1 and 3: Pemphigus vulgaris
  - Autoantibodies to desmoglein 3: Pemphigus vegetans.
  - Paraneoplastic pemphigus: It exhibits a more diverse antibody profile and involves desmogleins, plakins and other proteins.

II. Hailey-Hailey Disease

- **Clinical features:**
  - Autosomal dominant inheritance due to mutation in intracellular calcium pump (ATP2C1).
  - Intertriginous vesicles, crusts and erosions.
- **Histopathology:**
  - Suprabasal epithelium involves all the layers of the epithelium giving it a dilapidated brick wall appearance (Fig. 36.3).
  - Follicular epithelium is not involved.
  - Dermis shows a mild mixed nonspecific inflammation.
- **Immunofluorescence:** Negative for IgG, IgA, IgM and C3.

III. Keratosis Follicularis (Darier Disease)

- **Clinical features:**
  - Inherited in an autosomal dominant pattern due to mutation in an intracellular calcium pump (ATP2A2).

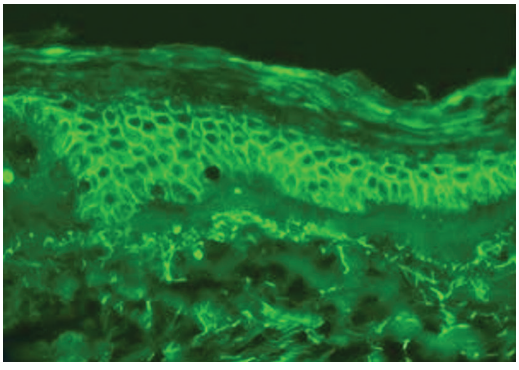

Fig. 36.2: Fishnet positivity for IgG and/or C3 on DIF

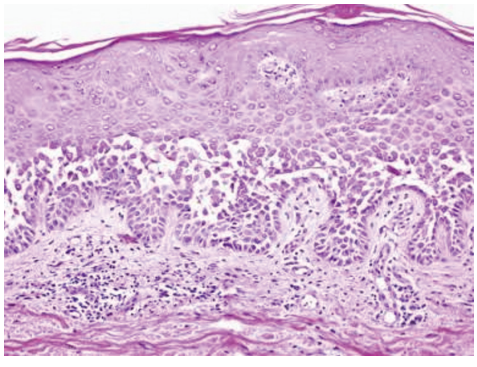

Fig. 36.3: Hailey-Hailey disease showing dilapidated brick wall appearance

- Usually presents as crusted scaly coalescent papules on the chest, axilla back and neck.

- **Histopathology:**
  - Suprabasal acantholysis with dyskeratotic keratinocytes.
  - Depending on the location they are given different names.
  - Stratum corneum: Known as grains
  - Stratum granulosum: Known as corps ronds
  - Also shows epidermal parakeratosis, irregular acanthosis and papillomatosis.
  - Mild to moderate lymphocytic infiltrate in the dermis.
- **Immunofluorescence:** Negative for IgG, IgA, IgM and C3.

IV. Transient Acantholytic Dermatosis (Grover Disease)

- **Clinical features:** Small erythematous pruritic papules on the chest and back.
- **Histopathology:**
  - Suprabasal acantholysis with associated dyskeratosis and parakeratosis.
  - The epidermal acantholysis is usually focal.
  - Closely resembles Darier disease and is distinguished from it by its clinical presentation.
- The differential diagnosis of vesiculobullous disorders showing suprabasal acantholysis is summarized in Table 36.4.

Table 36.4: Differential diagnosis for suprabasal acantholysis

	Pemphigus vulgaris	*Hailey-Hailey disease*	*Darier disease*	*Grover disease*
Suprabasal acantholysis	+	+	+	+
Full thickness acantholysis	–	+	–	–
Follicular epithelial involvement	+	–	–	–
Corp ronds and grains	–	–	+	–
Direct immunofluorescence	+ (Fish net pattern for IgG and C3)	Negative	Negative	Negative

V. Herpes Simplex and Herpes Zoster Infections

- **Clinical features:**
  - Grouped vesicles on an erythematous base.
  - These vesicles eventually form pustules, followed by crusts before resolving.
  - Shingles or recurrent Herpes simplex has a dermatomal distribution.
- **Histopathology:**
  - ***Epidermis:***
    Epidermal acantholysis along with characteristic changes in keratinocytes (3Ms):
    - Margination of the nuclear chromatin to the periphery
    - Multinucleation
    - Molding of nuclear membranes
  - Full-thickness epidermal acantholysis, epidermal necrosis and ulceration develop eventually.
  - ***Dermis:***
    - Dense inflammatory cell infiltrate which is predominantly neutrophilic.
    - May be accompanied by leucocytoclastic vasculitis.

- **Immunohistochemistry:** IHC for Herpes simplex and varicella zoster is useful in cases which lack the distinctive cytopathic changes.
- **Culture:** It is usually used for Herpes simplex virus from vesicles and pustules.
- **Tzanck smear:** Scrapings from the base of a vesicle or pustule are stained with Giemsa. Wright or Toluidine blue to view the characteristic nuclear changes in the exfoliated keratinocytes. Usually done in Varicella zoster.
- **Direct fluorescent antibody technique:** Also commonly used in the diagnosis of herpes virus infections.

SUBEPIDERMAL BULLAE WITH INFLAMMATION

I. Bullous Pemphigoid, Cicatricial Pemphigoid and Pemphigoid Gestationis (Herpes Gestationis)

- All three are caused by circulating antibodies directed against the basement membrane zone proteins.
- **Clinical features:**
  - Bullous pemphigoid (BP)
  - Large tense bullae on the flexor surfaces, trunk and intertriginous regions.
  - ***Cicatricial pemphigoid:***
    - Primarily involves the mucous membranes of the eyes and oral cavity.
    - It may also less commonly involve the pharyngeal, laryngeal, esophageal and genital mucous membranes.
    - Bullae are fewer in number compared to BP and heal with scarring.
  - ***Pemphigoid gestationis:*** Occurs exclusively in pregnant or post-partum women or women taking oral contraceptives.
- **Histopathology:**
  - Early lesions show subepidermal edema and eosinophilic spongiosis of the epidermis.
  - Well-developed lesion shows a sub-epidermal bulla (Fig. 36.4).
  - The bulla cavity may show eosinophils.
  - Dermal inflammatory infiltrate is admixed with numerous eosinophils
  - Cicatricial pemphigoid: In addition, scar is present in the superficial dermis underlying the bullae.

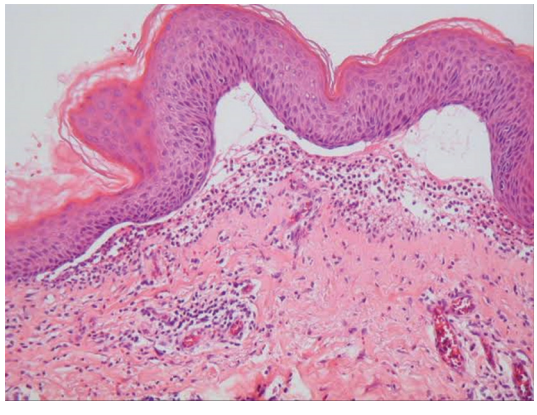

Fig. 36.4: Bullous pemphigoid showing a subepidermal bulla

- **Direct immunofluorescence:**
  - Linear deposits of IgG and C3 along the basement membrane zone in bullous pemphigoid and pemphigoid gestationis (Fig. 36.5).
- **Indirect immunofluorescence:**
  - Autoantibodies against 180-kd (BPAG2) and 230-kd (BPAG1) antigens.
  - Split skin indirect immunofluorescence shows immunoreactants aligning along the blister roof.
  - Cicatricial pemphigoid shows antibodies against laminin 5, Beta 4 Integrin and others in addition to BPAG1.

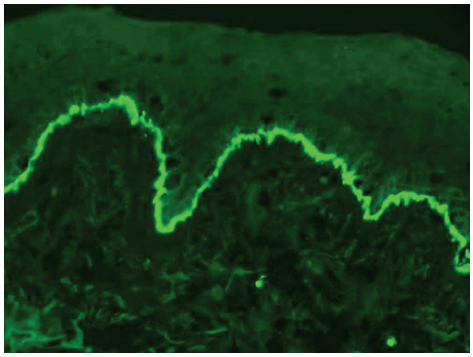

Fig. 36.5: Basement membrane positivity in bullous pemphigoid

II. Dermatitis Herpetiformis

- **Clinical features:**
  - Generally present on the scalp, extensor surfaces of the extremities and buttocks.
  - The lesions are extremely itchy and constant itching results in erosions, rather than intact vesicles.
  - Well-documented association with celiac disease.
- **Histopathology:**
  - Papillary dermis microabscesses are pathognomonic (Fig. 36.6).
  - Small subepidermal bullae coalesce to form a larger bulla.
  - Superficial dermis shows a predominantly neutrophilic infiltrate.
- **Direct immunofluorescence:** Granular deposits of IgA are seen in the papillary dermis of perilesional skin (Fig. 36.7).

III. Linear IgA Bullous Dermatosis

- **Clinical features:**
  - Linear distribution of bullae on an erythematous base.
  - Sites involved are: Thighs, buttocks, lower trunk, scalp, face and genitals.
  - Associated with vancomycin.

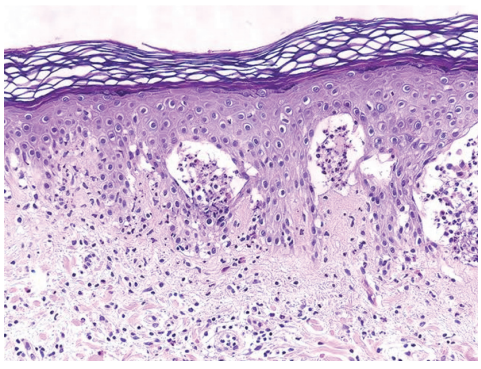

Fig. 36.6: Dermatitis herpetiformis showing papillary dermis microabscesses

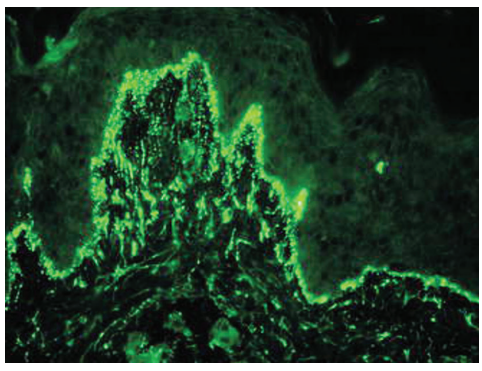

Fig. 36.7: Granular deposits of IgA in the papillary dermis

- **Histopathology:**
  - Subepidermal bulla with a neutrophilic infiltrate.
- **Direct immunofluorescence:**
  - Linear IgA deposits along the dermoepidermal junction, in contrast to the granular deposits in dermatitis herpetiformis.

IV. Epidermolysis Bullosa Acquisita

- **Clinical features**

  Acquired autoimmune blistering disorders with 2 clinical variants:
  - ***Classical (mechanobullous) variant:*** In this blisters develop on the hands and feet and other areas subjected to trauma.
  - ***Inflammatory variant:*** Large tense blisters that show a predilection for the flexural and intertriginous areas.
- **Histopathology:**
  - ***Classical variant:*** Pauci-cellular subepidermal bulla.
  - ***Inflammatory variant:*** subepidermal bulla with a neutrophilic infiltrate.
- **Immunofluorescence:**
  - Direct immunofluorescence findings are identical to those of bullous pemphigoid, therefore indirect fluorescence (salt-split skin) is required to make the diagnosis.
  - Split skin immunofluorescence shows immunopositivity along the floor of the bulla whereas bullous pemphigoid shows it on the roof.

SUBEPIDERMAL BULLA WITHOUT INFLAMMATION

I. Epidermolysis Bullosa

- **Clinical features:**
  - It is a group of inherited disorders of structural proteins in the skin.
  - Based on the location of the affected protein it is further classified as follows:
    - Lower epidermis (EB simplex)
    - Basement membrane (junctional EB)
    - Upper dermis (dystrophic EB)
- **Histopathology:**
  - Subepidermal bulla with an intact epidermis and minimal inflammation in the bulla.
  - Superficial dermis shows scarring in dystrophic types of EB.
- **Immunofluorescence:** It shows basement membrane zone positivity.

II. Porphyria Cutanea Tarda

- **Clinical features:**
  - Blisters lead to small angular scars, changes in pigmentation or hypertrichosis.
  - Areas exposed to sunlight are commonly involved
  - Porphyria cutanea tarda is caused by decreased activity of uroporphyrinogen decarboxylase enzyme.
- **Histopathology:**
  - Non-inflammatory subepidermal bullae

- Dermis may show the following:
  - ***Festooned pattern:*** Projection of dermal papillae into the bulla
  - ***Caterpillar bodies:*** PAS-positive, diastase-resistant linear globules at the roof of the bulla
- Basement membrane of the epidermis and the vessels show PAS-positive eosinophilic deposits.

- The DIF, indirect immunofluorescence and salt-split skin immunofluorescence findings are summarized in Tables 36.5 to 36.7.

Table 36.5: DIF findings in vesiculobullous disorders

Vesiculobullous disorders	*DIF findings*
Pemphigus vulgaris	Epidermal intercellular deposition of IgG (IgG1 and G4)—chicken wire or fishnet appearance
Paraneoplastic pemphigus	Intercellular deposit of IgG (+C3) along with granular or linear basement membrane deposit of IgG, C3 + IgM
Pemphigus herpetiformis	Epidermal intercellular deposit of IgG + C3
Cicatricial pemphigoid	Linear deposit of C3, IgG and IgA at dermoepidermal junction (DEJ)—shoreline appearance
Bullous pemphigoid	Linear DEJ deposit of IgG and C3
Epidermolysis bullosa acquisita	Linear DEJ deposit of IgG and C3 (C3 is deposited to a lesser extent)
Linear IgA dermatosis	Linear DEJ deposit of IgA
Dermatitis herpetiformis	Granular DEJ deposit of IgA
Erythema multiforme	Granular deposits of IgG, C3, IgM and fibrinogen around the dermal vessels and/or DEJ
Systemic lupus erythematosus	Shaggy or granular basement membrane zone positivity for IgG, C3 or IgM—positive lupus band test

Table 36.6: Indirect IF findings in vesiculobullous disorders

Vesiculobullous disorders	*Indirect IF findings on monkey/ guinea pig esophagus*
Pemphigus vulgaris and Pemphigus foliaceous	Intercellular deposition of circulating IgG antibodies in the epidermis
Paraneoplastic pemphigus	Intercellular epidermal deposition + DEJ deposition of circulating IgG
Bullous pemphigoid	Circulating IgG autoantibodies against the basement membrane zone proteins
Epidermolysis bullosa aquisita	Circulating IgG autoantibodies against the basement membrane zone proteins

Table 36.7: Indirect IF findings by salt-split technique

Vesiculobullous disorder	*Findings by salt-split technique*
Bullous pemphigoid	IgG autoantibodies bind to blister roof
Epidermolysis bullosa acquisita	IgG autoantibodies bind to blister floor
Linear IgA technique	IgA deposition either on blister roof or floor

POINTS TO REMEMBER

- Vesiculobullous disorders comprise a large group of clinically heterogeneous disorders.
- They can be potentially life-threatening if not managed properly.
- A systematic, ''top-down'' histopathologic approach can help simplify the evaluation of these diseases.
- In the approach to these following three points hold the key to diagnosis:
  1. Site or level of the blister.
  2. Findings that implicate the mechanism of blister formation.
  3. The type of inflammation when present.
- Special attention must be paid to biopsy technique, clinical stage of disease, age of the biopsied blister, and immunofluorescence findings.
- Effective communication between clinician and pathologist is critical for correct diagnosis.

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Contemporary Gleason Grading of Prostatic Carcinoma

37

INTRODUCTION

- Prostate cancer is the second most common cancer and the sixth leading cause of cancer death among men worldwide.
- In India, it is among the top ten most common cancers in Indian males as per the ICMR and various state cancer registries.
- The incidence rate in India is 9–10/100000 population which is higher than other parts of Asia and Africa but lower than the USA and Europe.
- It is the second leading site of cancer among males in large Indian cities like Delhi, Kolkata, Pune and Thiruvananthapuram; the third leading site of cancer in cities like Bengaluru and Mumbai.
- The incidence rate of this cancer is constantly and rapidly increasing in all the Population-Based Cancer Registries (PBRCs).
- According to a study by the International Agency for Research on Cancer, the worldwide Prostate Cancer burden is expected to grow to 1.7 million new cases and 4,99,000 new deaths by 2030 due to the growth and aging of the population.
- To discuss the issues related to prostate health in India, September/October is observed as Prostate Health Month across the country.
- Previously it was thought, that the prevalence of prostate cancer in India was far lower as compared to Western countries.
- But with the increased migration of rural population to the urban areas, changing lifestyles, increased awareness, and easy access to medical facilities, more cases of prostate cancer are being picked up and it is coming to the knowledge that we are not very far behind the Western countries.
- In autopsy studies of men who died of other causes, more than 20% of men aged 50 to 59 years and more than one-third of men aged 70 to 79 years were found to have prostate cancer.
- The median age of death from prostate cancer is 80 years and more than two thirds of all men who die of prostate cancer are older than age 75 years.

RISK FACTORS

- **Age:** With increasing age the risk for prostate cancer also increases.
- **Race:** African Americans have a higher incidence and more than twice the cancer mortality rate as compared to Caucasians.

- **Family history:** Increased with family history, double the risk if one first-degree relative is affected, and quadruple the risk if more than two first-degree relatives are affected.
- **Diet:** Red meats, fatty diet increase risk and cruciferous vegetables, lycopene (found in tomatoes), Vitamin E and selenium offer protective effects.
- Most cancers develop in the peripheral zone which is area palpated by digital rectal examination (DRE).
- The vast majority of these tumors, i.e. >95% are adenocarcinomas.
- The remaining 5% is constituted by squamous cell carcinoma, transitional cell carcinoma and carcinosarcoma.
- The tumor cells express androgen receptors and depend on androgens for growth.

Clinical Presentation

- Localized prostate cancer is asymptomatic, usually discovered by the detection of a suspicious nodule on DRE or elevated serum PSA level.
- Most prostatic cancers arise peripherally away from the urethra, and therefore urinary symptoms occur late.
- Patients with clinically advanced prostatic cancer may present with urinary symptoms.
- New onset impotence indicates periprostatic involvement.
- Common symptoms and signs are as follows:
  - Urinary urgency, frequency, hesitancy
  - Nocturia
  - New onset impotence
  - Back pain
  - Acute urinary retention
  - Spinal cord compression
  - Rarely supraclavicular lymphadenopathy or hepatic abnormalities.

Screening Guidelines of the United States Preventive Services Task Force (USPSTF)

Men ages 55 to 69 years

- The USPSTF recommends that clinicians inform men age 55 to 69 years about the potential benefits and harms of prostate-specific antigen (PSA)-based screening for prostate cancer.
- The decision about whether to be screened for prostate cancer should be an individual one.
- Screening offers a small potential benefit of reducing the chance of dying of prostate cancer.
- However, many men will experience potential harms of screening, including false-positive results that require additional testing and prostate biopsy.
- Overdiagnosis, overtreatment and treatment complications, such as incontinence and impotence may occur.
- The USPSTF recommends individualized decision-making about screening for prostate cancer after discussion with a clinician, so that each man has an opportunity to understand the potential benefits and harms of screening and to incorporate his values and preferences in his decision.

Men age 70 years and older

- The USPSTF recommends against PSA-based screening for prostate cancer in men age 70 years and older.

Benefits of Early Detection and Treatment

- The goal of screening for prostate cancer is to identify high-risk, localized prostate cancer that can be successfully treated, thereby preventing the morbidity and mortality associated with advanced or metastatic prostate cancer.
- Evidence from randomized clinical trials shows that PSA-based screening programs in men ages 55 to 69 years may prevent up to 1 to 2 deaths per 1,000 men screened.
- Screening programs prevent up to 3 cases of metastatic prostate cancer per 1,000 men screened.
- Adequate evidence from randomized clinical trials is consistent with no mortality benefit of PSA-based screening for prostate cancer in men aged 70 years and older.

SCREENING METHOD

- After discussion, men who want to be screened should be tested with a PSA test.
- The digital rectal exam (DRE) may also be done as a part of screening.
- If no prostate cancer is found as a result of screening, the time between future screenings depends on the results of the PSA blood test as follows:
  - PSA of less than 2.5 ng/mL needs to be re-tested every 2 years.
  - PSA level is 2.5 ng/mL or higher screening should be done yearly.
- Because prostate cancer usually grows slowly, men without symptoms of prostate cancer who do not have a 10-year life expectancy should not be offered testing since they are not likely to benefit.

Diagnosis

- Established by TRUS-guided needle biopsy—gold standard.
- Clinical evaluation also includes an MRI of abdomen/pelvis which helps to evaluate visceral organs, urinary tract, para-aortic and pelvic lymphadenopathy.
- Metastatic disease evaluation involves a bone scan in conjunction with plain radiographs for suspicious areas, especially if PSA >10 and high Gleason grade

Prostate Biopsy

- The histopathological examination of biopsy material obtained by ultrasound-guided transrectal 18-gauge core biopsy has become the standard of care.
- Sextant biopsy scheme: The original systematic biopsy method is the sextant biopsy scheme (1 core from the base, mid, and apex bilaterally).
- However, it was seen that up to 30% of cancers were missed by the standard sextant biopsy.
- Thus, an extended biopsy scheme of 10 to 12 cores (Fig. 37.1) is now recommended to optimize the ratio of cancer detection to adverse post-biopsy events.
- Lesion-guided biopsy can be added to further optimize cancer detection.

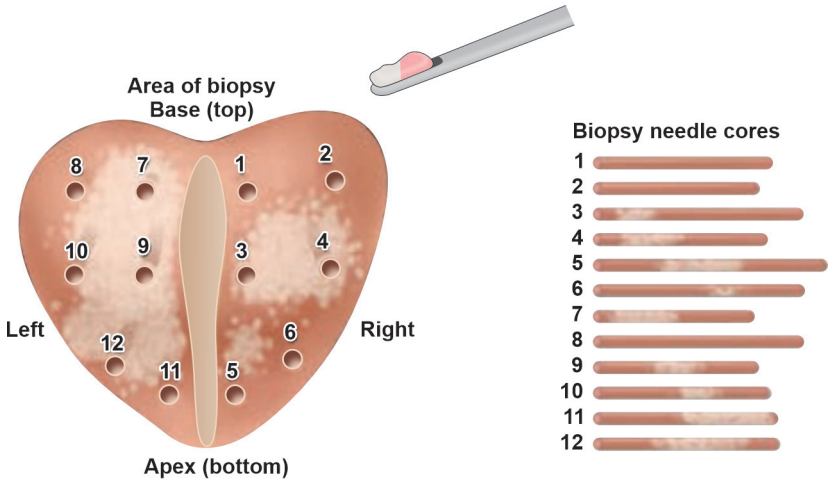

Fig. 37.1: Extended prostate biopsy scheme

Repeat Biopsies in Case of High-grade Prostatic Intraepithelial Neoplasia (HGPIN) and Atypical Small Acinar Proliferation (ASAP)

HGPIN

- High-grade prostatic intraepithelial neoplasia is thought to be a precursor to invasive Adenocarcinoma.
- During the sextant biopsy scheme era, the cancer detection rate on repeat biopsy for HGPIN was 25–70%.
- With the introduction of extended biopsies, the cancer detection rates on the first repeat biopsy for HGPIN has decreased to 2.3–4.5% because of extensive sampling in the first instance.
- In the current era of extended biopsy schemes, HGPIN is no longer considered a strict indication for repeat biopsy and patients should be followed clinically with PSA and digital rectal examination (DRE).

ASAP

- ASAP finding should be viewed differently than HGPIN.
- Atypical small acinar proliferation is a focus of morphologic malignant cells with the presence of a basal cell layer.
- Cancer detection rates on repeat biopsy for ASAP found on sextant biopsies is 40% to 50%.
- Using an extended core biopsy scheme, the cancer detection rate remains as high as 36% to 59.1% on the first repeat biopsy and 16% on the second repeat biopsy.
- Thus, the final recommendations are as follows:
  - Atypical small acinar proliferation lesions are cancerous until proven otherwise and should undergo repeat biopsy.
  - Repeat biopsy may no longer be indicated for HGPIN lesions in the era of extended core biopsy unless the patient has an increase in PSA or change in DRE.

MAGNETIC RESONANCE IMAGING (MRI)-TARGETED PROSTATE BIOPSIES

- MRI-targeted biopsy, compared with the standard extended biopsy has a higher rate of detection of clinically significant prostatic carcinoma with fewer biopsy cores.
- It also provides better representation of the tumor characteristics such as tumor volume and Gleason grade.
- Targeted biopsy preferentially detects higher-grade prostate cancer while missing lower-grade tumors.
- These findings suggest that MRI-targeted biopsy can improve the biopsy and resection specimen Gleason score concordance.
- Therefore, it will enhance the prognostic significance of Gleason score derived from the biopsy specimens.

Gleason Grading

- This system was devised by *Donald F. Gleason* (Nov 20, 1920–Dec 28, 2008), an American physician and pathologist in the year 1966.
- It remains the cornerstone for the management of prostate cancer.
- He was chief of pathology at the Minneapolis VA Medical Center and received three degrees from and taught at the University of Minnesota
- He devised grades of 1 to 5, based on glandular architecture at low to medium power, that were shown to predict outcomes in prostate cancer.
- The grading system has undergone continuous modification and changes in response to changes in the clinical practice of diagnosis and treatment of prostate cancer since its inception.
- The most significant changes were introduced in 2005 by the International Society of Urological Pathology (ISUP) and further modification was done in 2014.
- In March 2005, a consensus conference was summoned by the International Society of Urological Pathology (ISUP) in San Antonio, Texas, USA, in an effort to standardize and unify the use of the Gleason grading system.
- The architectural patterns according to the 2005 ISUP modified Gleason system are as follows:
  - ***Pattern 1:*** Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini.
  - ***Pattern 2:*** Like Pattern 1, fairly circumscribed, yet at the edge of the tumor nodule there may be minimal infiltration. Glands are more loosely arranged and not quite as uniform as Pattern 1.
  - ***Pattern 3:*** Discrete glandular units, typically smaller glands than seen in Gleason patterns 1 or 2. Infiltrates in and amongst non-neoplastic prostate acini. Marked variation in size and shape. Smoothly circumscribed small cribriform nodules of tumor.
  - ***Pattern 4:*** Fused microacinar glands. Ill-defined glands with poorly formed glandular lumina. Large cribriform glands. Irregular cribriform glands. Hypernephromatoid.
  - ***Pattern 5:*** Essentially no glandular differentiation, composed of solid sheets, cords or single cells. Comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses.

Problems with the 2005 Gleason System

- Scores 2–5 are currently no longer assigned.
- Certain patterns that originally Gleason defined as a score of 6 are now graded as 7, thus leading to contemporary Gleason score 6 cancers having a better prognosis than historic score 6 cancers.
- In practice the lowest score is now assigned a 6, although it is on a scale of 2–10.
- This leads to a logical yet incorrect assumption on the part of patients that their cancer is in the middle of the scale, compounding the fear of their cancer and the belief that the cancer is serious and the treatment is necessary.

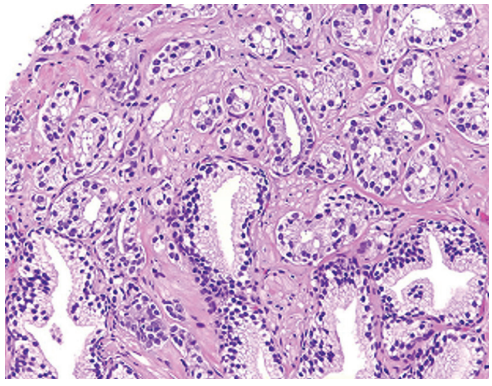

Fig. 37.2A: Malignant glands infiltrating between benign glands

New Gleason Grading System (2014 ISUP)

- The most important change in the 2014 modification is the strict definition of each pattern.
- Gleason patterns 1 and 2: Due to its misleading clinical implications, a Gleason score (GS) of 2 to 4 should not be rendered in needle biopsies.
- The major limitation of diagnosing GS 4 on needle biopsy is that one cannot see the entire edge of the lesion to determine if it is circumscribed.
- Therefore, from practical standpoint Gleason pattern (GP) in contemporary practice starts at 3 and GS starts at 6 in prostate biopsy specimens and most TURP and RP specimens.

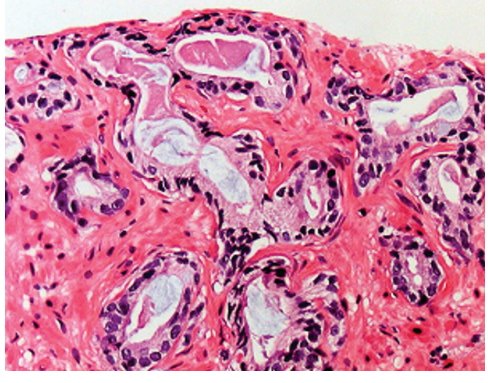

Fig. 37.2B: U- and Y-shaped glands

Gleason Pattern 3

- Pattern 3 represents the most common GP encountered in prostate needle biopsy.
- It is strictly defined as discrete, well-formed malignant glands.
- Variably sized individual glands infiltrating between benign glands represent the classical example of GP 3.
- Prostate cancer may demonstrate branching such as "U" or "Y"-shaped glands, which is not considered as fusion and should be regarded as GP 3.
- Crowded microacinar glands are also GP 3.

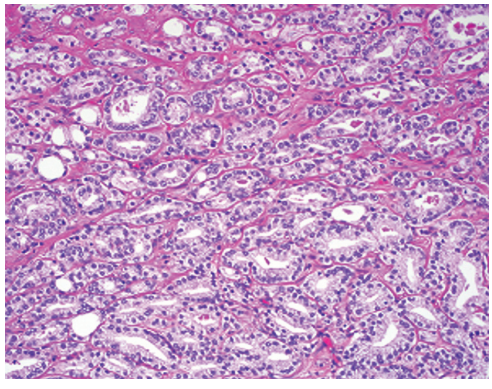

Fig. 37.2C: Crowded microacinar pattern

Gleason Pattern 4

- It was first described as large clear cells growing in a diffuse pattern resembling hypernephroma.
- GP 4 was later expanded to include raggedly infiltrating, fused-glandular tumor (Fig. 37.3A) and glands that are not single and separate, but coalesce and branch.
- 2005 ISUP consensus —GP 4 includes ill-defined glands with poorly formed glandular lumens in addition to the previously recognized patterns:
  - Fused microacinar glands
  - Large cribriform glands
  - Cribriform glands with an irregular border, and hypernephromatoid cells.
- 2014 ISUP consensus: It recognizes cribriform, fused and poorly formed glands.
- However, the term "hypernephromatoid" should not be used.
- A major departure in the 2014 ISUP modified Gleason grading system from the original system is that all cribriform carcinomas without comedonecrosis are considered GP 4.
- All cribriform prostatic carcinoma (Fig. 37.3B), regardless of its shape, contour and size, should be interpreted as GP 4.
- Poorly formed glands (Fig. 37.3C) represent Gleason pattern 4.
- Glands with no or barely visible lumens are considered "poorly formed".
- Poorly formed glands that are adjacent to other well-formed small glands likely result from tangential sectioning and therefore should not be graded as a pattern 4—graded as GP 3.
- Five or less poorly formed glands in a cluster regardless of their location should not be graded as GP 4.
- Minimum number of poorly formed glands in a focus to be graded as Gleason pattern 4 is 6.

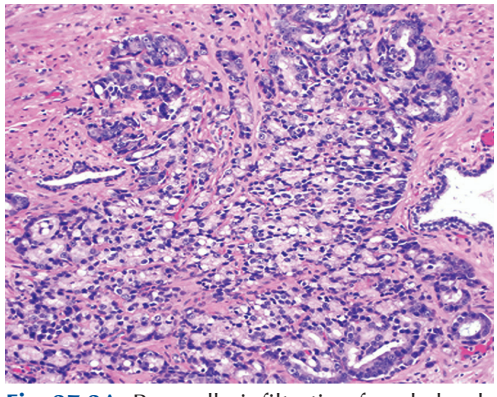

Fig. 37.3A: Raggedly infiltrating fused glands

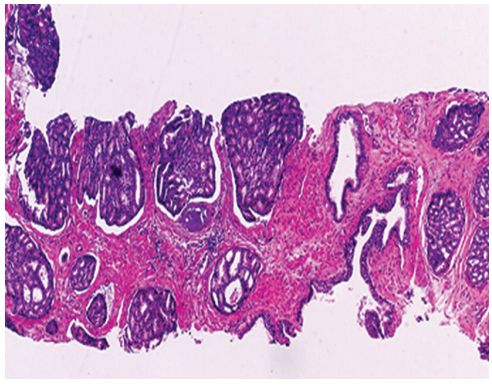

Fig. 37.3B: Cribriform carcinoma

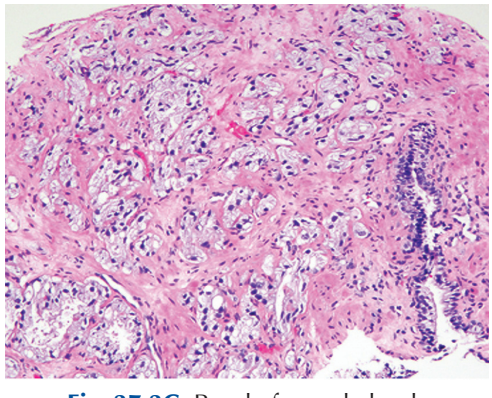

Fig. 37.3C: Poorly formed glands

Gleason Pattern 5

- The morphological spectrum of GP 5 is wide.
- It includes prostate carcinoma exhibiting no glandular differentiation, solid tumor sheets, cords, or single cells and comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses.

- It has the worst biological behavior.
- Histological features that are diagnostic of Gleason pattern 5 prostate cancer include large tumor nests (>20 cells within the nest, Fig. 37.4A).
- Single cells/cords (Fig. 37.4B).
- Small to medium-size nests should not be classified as pattern 5 due to poor reproducibility.
- Intraluminal coagulative necrosis is required to make the diagnosis of comedocarcinoma (pattern 5).
- Signet ring cells in single cells or within nests is pattern 5.

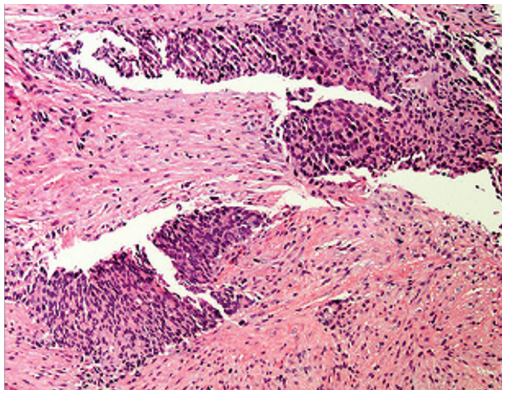

Fig. 37.4A: Large tumor cell nests

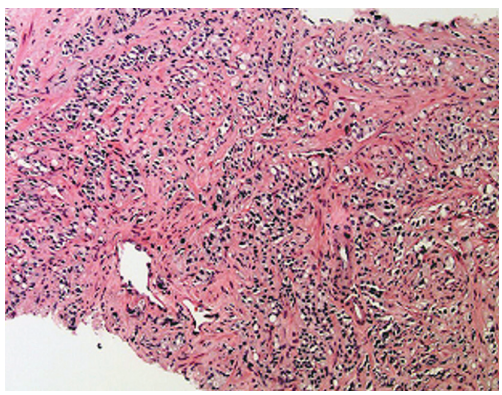

Fig. 37.4B: Tumor cells present singly and in cords

Grading Prostate Cancer with Unusual Morphologies and Patterns

- Foamy gland carcinoma: Graded on the basis of underlying architecture.
- Collagenous micronodules:
  - These are hypo- or acellular collagenous substance found within or immediately adjacent to cancer glands.
  - Usually, they fall under pattern 3. But the final grade is done on the basis of underlying architecture.
- Glomeruloid structures are intraglandular proliferation of cancer cells that superficially resemble renal glomeruli.
- They are considered a morphological continuum to cribriform glands and therefore are regarded as GP 4.
- Mucinous (colloid) carcinoma: It consists of tumor glands within abundant extravasated mucin occupying >25% of the tumor.
- These tumors should be graded based on the underlying architecture.
- Signet ring cell-like carcinoma: It is graded as pattern 5.
- Small cell carcinoma, adenosquamous, squamous cell and sarcomatoid carcinoma are not graded at all.

Operational Changes in Gleason Scoring

Grading a Biopsy Specimen with Multiple Cores Positive for Cancer of Different Gleason Grades

- An important issue in grading a biopsy with prostate carcinoma of different Gleason scores involving multiple cores is whether to render a global Gleason score or the worst score.
- The ISUP consensus recommends assigning Gleason score to individual cores as long as they are submitted in separate containers or their locations are specified by urologists.

- When multiple cores are submitted in a container without site specification—Gleason score of individual cores or worst Gleason score should be reported.
- In cases where a container contains multiple pieces of tissue and one cannot be sure if one is looking at an intact core or broken bits—overall Gleason score for that container should be provided.
- Any amount of high grade in the needle biopsy should be reported.
- In needle biopsy—high-grade (GP 4 and 5) tumor of any quantity should be included in the final Gleason score.
- For example, if a biopsy specimen includes tumors with patterns 3, 4, and 5 in various proportions.
- In such a tumor, both the primary and the highest pattern should be recorded and added together to get the final Gleason score.
- If pattern 3 is the most common pattern and pattern 5 the highest pattern, the final Gleason score would be 3 + 5 = 8.

Grading Radical Prostatectomy Specimens

- In radical prostatectomy specimen even a limited amount of high-grade tumor should be reported as either secondary (when it is >5% of the tumor) or tertiary pattern (when it is <5% of the tumor).
- In radical prostatectomy with multifocal cancer with heterogeneity in Gleason score (GS) and genetic alterations.
- For these cases the concept of dominant/index nodule was adopted for the convenience of reporting Gleason score of the entire case.
- It was believed that the dominant nodule is the largest tumor nodule and has the highest stage and grade.
- However, it was observed that in a significant number of cases the largest tumor volume, highest GS and staging parameters do not always concur in the same tumor nodule.
- In these cases, it was recommended that pathologists should de-emphasize the concept of dominant tumor nodule.
- Multifocal nature of the disease should be emphasized and the pathological features of all independent tumor foci should be documented.

PROGNOSTIC GLEASON GRADE GROUPING

- GS is often grouped into three tiers (6, 7, 8–10) for prognostic and therapeutic purposes, despite the fact that GS 3 + 4 vs 4 + 3 and 8 vs 9–10 have significantly different prognosis.
- To address this issue, nine potential Gleason scores (2–10) were grouped into five groups.
- These grade groups were also validated on biopsy correlating with the risk of progression after radical prostatectomy.
- The various grade groups and their risk of relapse 5 years following radical prostatectomy are presented in Table 37.1.

Table 37.1: Gleason grade groups and their risk of relapse 5 years following radical prostatectomy		
Group 1	Gleason score 6	5%
Group 2	Gleason score 3 + 4 = 7	17%
Group 3	Gleason score 4 + 3 = 7	35%
Group 4	Gleason score 4 + 4 = 8	37%
Group 5	Gleason score 9–10	76%

POINTS TO REMEMBER

- The new Gleason grading system for prostate cancer (ISUP 2014) has obvious benefits.
- It gives a more accurate grade stratification than the current Gleason system.
- A simplified grading system of five grade groups in contrast to multiple possible scores depending on various Gleason pattern combinations.
- Lowest grade is 1 as opposed to the current practice of Gleason score 6, with the potential to reduce overtreatment of indolent prostate cancer.
- The new grading system, using the above terminology, has been accepted by the 2016 World Health Organization (WHO) classification of prostate tumors.
- To avoid confusion and in order to make the clinicians familiar with the new system, it will be prudent to report the new grading system, in conjunction with the previous Gleason system, until it becomes widely accepted and practiced [i.e. Gleason score 3 + 3 = 6 (Grade Group 1)].

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Approach to the Diagnosis of Cutaneous Lymphomas

38

Anisha Manocha

INTRODUCTION

- The group of primary cutaneous lymphomas (CL) are non-Hodgkin lymphomas.
- They present in the skin without extracutaneous disease at the time of diagnosis.
- CL exhibits distinct clinical, histological, immunophenotypic, and genetic features.
- They differ in prognosis and treatment from systemic lymphomas with similar histological features.
- To reflect the characteristics of CL, the WHO-EORTC classification for CL was introduced in 2005.
- Its most recent updated form was published in 2018 shown in Table 38.1.
- There are two major concerns in case of a biopsy with a dermal lymphocytic infiltrate:
  - Whether the infiltrate is neoplastic or reactive
  - Assigning the correct diagnostic category according to the WHO/EORTC classification.

Table 38.1: WHO/EORTC (2018) classification of cutaneous lymphomas

Cutaneous T cell lymphomas
Mycosis fungoides (MF)
MF variants
⊥ Folliculotropic MF
⊥ Granulomatous slack skin
⊥ Pagetoid reticulosis
Sézary syndrome
Adult T cell leukemia/lymphoma
Primary cutaneous CD30-positive lymphoproliferative disorders
⊥ Lymphomatoid papulosis
⊥ Primary cutaneous anaplastic large cell lymphoma
Subcutaneous panniculitis-like T cell lymphoma
Primary cutaneous peripheral T cell lymphoma, rare subtypes
⊥ Primary cutaneous γ/δ T cell lymphoma
⊥ Primary cutaneous aggressive epidermotropic CD8-positive T cell lymphoma (provisional)
⊥ Primary cutaneous CD4+ small/medium-sized pleomorphic T cell lymphoproliferative disorder (provisional)
⊥ Primary cutaneous acral CD8+ T cell lymphoma (provisional)

Contd.

Table 38.1: WHO/EORTC (2018) classification of cutaneous lymphomas
Primary cutaneous peripheral T cell lymphoma, not otherwise specified
Extranodal NK/T cell lymphoma, nasal type
Chronic active EBV infection
Cutaneous B cell lymphomas
Primary cutaneous marginal zone B cell lymphoma
Primary cutaneous follicle center lymphoma
Primary cutaneous diffuse large B cell lymphoma, leg type
EBV-positive mucocutaneous ulcer (provisional)

CUTANEOUS T CELL LYMPHOMAS (CTCL)

Mycosis Fungoides (MF)

- MF is the most common primary cutaneous T cell lymphoma.
- It is derived from a mature post-thymic peripheral T cell.
- Clinical presentation:
  - There is generally a preceding history of contact dermatitis.
  - Early manifestation of the disease is known as parapsoriasis.
  - Long-standing any erythematous scaly patch/plaque over the trunk.
  - It may progress to a generalized infiltrated plaque or a tumor.
- *Sézary syndrome* manifests with generalized erythroderma with lymphadenopathy and circulating atypical lymphocytes.
- *D'emblee variant* shows cutaneous tumors which do not proceed via the prolonged superficial phase.

Microscopy

- **Early stage/parapsoriasis**
  - There is the presence of parakeratosis and acanthosis.
  - Mild superficial dermal perivascular or band-like infiltrate.
- **Patch stage**
  - Basilar tagging by lymphocytes (epidermotropism).
  - *Pautrier's microabscesses:* Lymphocytes permeate the epidermis in couplets or triplets; characteristically in the absence of spongiosis.
  - Intraepidermal lymphocytes show lacunae around them known as *Haloed Cells.*
  - Lymphocytes show irregular or cribriform nuclei.
  - Papillary dermal collagen becomes thickened known as wiry collagen.
- **Plaque stage**
  - Lymphoid infiltrate becomes increasingly dense and band-like in the superficial dermis.
  - Increased proportion of intermediate and large atypical lymphocytes with a higher degree of atypia.
- **Tumor stage**
  - Increased number of highly atypical large lymphocytes.
  - Epidermal involvement diminishes.
  - May transfer into large cell lymphoma.

IHC

- CD4+, CD8 –/diminished leading to high CD4:CD8 ratio.
- CD2+, CD3+, CD5+, TCRB+
- An important finding is CD7 dropout leading to diminished number of CD7 positive cells.
- CD7 dropout and T cell receptor gene rearrangement are highly specific for MF.

Variants of MF

- **Folliculotropic MF (FMF)**
  - It is a distinct variant of MF accounting for approximately 10% of all MF cases.
  - It is characterized by predominantly folliculotropic infiltrates (i.e. exocytosis of tumor cells into the hair follicle epithelia).
  - The advanced form of FMF with thicker alopecic plaques and deep, dense lymphocytic infiltrates has an aggressive course and needs to be treated more intensively.
  - The early FMF form with follicular papules, acneiform lesions with comedones and cysts, and alopecic patches run an indolent course.
- **Granulomatous slack skin**
  - It is regarded as a distinct variant of MF based on its characteristic clinical presentation of bulky skin folds, particularly in the intertriginous folds.
- **Pagetoid reticulosis**
  - This is a variant of MF with an excellent prognosis.
  - It is characterized by a solitary, slowly growing psoriasiform lesion often located on acral sites.
  - Histologically a prominent epidermotropic lymphocytic infiltrate is seen.

Differential Diagnosis

- **Chronic reactive inflammatory dermatoses:** MF is favored by:
  - Epidermotropism
  - Haloed cells
  - Basilar tagging of lymphocytes

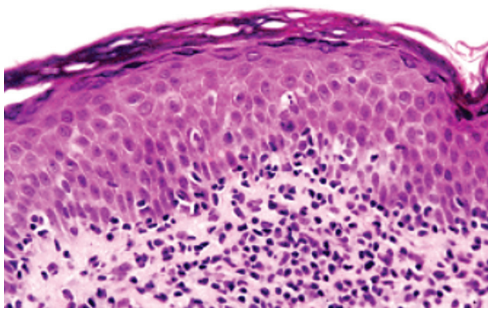

Fig. 38.1: Papillary dermal interstitial infiltrate along with basilar tagging (epidermotropism)

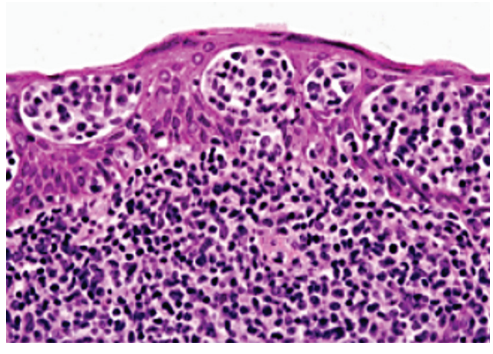

Fig. 38.2: Plaque stage showing multiple prominent clusters of atypical lymphocytes in the epidermis

- **Sézary syndrome is favored by the triad:**
  - Erythroderma
  - Lymphadenopathy
  - Atypical circulating T cells in peripheral blood
- At least 100 atypical cells (sezary cells) /cumm of blood should be present.
- It has an aggressive clinical course.
- IHC can also help to differentiate as shown in Table 38.2.

Table 38.2: Comparison of IHC between MF and Sézary syndrome

Mycosis fungoides	*Sézary syndrome*
CCR7 +	CCR7–
L-Selectin +	L- Selectin–
CD27 +	CD27–
CCR4 +	CCR4–

Histopathology

- Significant parakeratosis and acanthosis are seen.
- Subepidermal band-like infiltrate of lymphocytes with cerebriform nucleus.
- Epidermotropism and basilar tagging by lymphocytes is less prominent as compared to MF.
- Lymph node infiltration by Sézary cells may be seen.

IHC

- CD4+ (marker of helper T cells)
- CD7 dropout may occur
- T cell receptor gene rearrangement is always present.

SUBCUTANEOUS PANNICULITIS-LIKE T CELL LYMPHOMA (SPTCL)

- It is defined by the following features:
  - Primarily infiltration of the subcutaneous tissue.
  - High-grade cytologic features are present.
- T cells with a cytotoxic phenotype.
- Rare, comprising <1% of all non-Hodgkins lymphoma.
- Clinically present with deep-seated variable-sized nodules on extremities or trunk.
- Histopathology shows a diffuse cellular infiltrate involving the septae and lobules of subcutis.
- The tumor cells consist of small lymphocytes along with large transformed cells having a hyperchromatic nucleus.
- They show characteristic rimming around individual adipocytes.
- IHC: The tumor cells are CD8+
- SPTCL is defined by the expression of TCR alpha/beta by the tumor cells.
- On the other hand, expression of TCR gamma/delta is seen in the subcutaneous form of gamma/delta T-cell lymphoma.
- In most patients, the disease runs an indolent course.
- Exception being a few patients developing hemophagocytic syndrome linked to an aggressive course with high mortality.

- Pathogenetically SPTCL carries features of a hyperactive immune reaction.
- This may explain the fact that immunomodulating therapies are effective in these patients.

PRIMARY CUTANEOUS ANAPLASTIC LARGE CELL LYMPHOMA AND LYMPHOMATOID PAPULOSIS (CD 30 POSITIVE LYMPHOPROLIFERATIVE DISORDERS)

Both show cutaneous infiltration by CD30 + cells.

PRIMARY CUTANEOUS ALCL

- Clinically present as a single violaceous nodule or multiple grouped nodules confined to one body site.
- **Histopathology:**
  - Dense dermal infiltrate of large pleomorphic anaplastic tumor cells. Cohesive aggregates may be seen.
  - These cells have irregular nuclei, prominent nucleoli and abundant pale eosinophilic cytoplasm.
  - Mitoses are increased.
- **IHC:**
  - CD 30 +
  - CD2, 3, 4, CD45 RO+
  - ALK-1 is characteristically negative.
- T cell receptor rearrangement is seen.
- It is extremely crucial to differentiate primary cutaneous ALCL from systemic ALCL involving the skin.
- This is imperative because systemic ALCL has an adverse prognosis as it responds poorly to chemotherapy.
- Primary cutaneous ALCL, on the other hand, is an indolent disease.

LYMPHOMATOID PAPULOSIS

- Clinically present with recurrent crops of papulonodules over trunks and extremities in adults which ulcerate and heal with varioliform scars.
- About 10–20% cases develop lymphomas, e.g. Hodgkin, MF and ALCL.
- **Microscopy:**
  - Wedge-shaped dermal infiltrate with the base near the epidermis.
  - Epidermis shows ulceration, necrosis and exocytosis.
  - The cases are divided into five histologic types:
    1. Type A—resembles HL
    2. Type B—resembles MF
    3. Type C—resembles ALCL
    4. Type D—marked epidermotropism composed of neoplastic cells expressing CD8 and CD30
    5. Type E —ulcerated lesions with angioinvasion and angiodestruction CD30+ and CD8+.
- **IHC:**
  - CD4+, CD8– (exceptions are type D and E)
  - CD30+ (in anaplastic cells in type A and C)
- T cell receptor gene rearrangement may be seen in 60–70% cases.

Differential Diagnosis

1. *Pityriasis lichenoides et varioliformis acuta (PLEVA):* It lacks significant cytologic atypia.
2. *Cutaneous Hodgkin lymphoma:* It will show CD30+, CD15+ and CD45RO – Reed: Sternberg cells (cells of lymphomatoid papulosis are CD 30+, CD15 – CD45RO+)

CD4+ SMALL/MEDIUM-SIZED T CELL LYMPHOPROLIFERATIVE DISORDER (CD4+SMT-LPD)

- It is listed as a provisional entity.
- CD4+SMT-LPD is an indolent neoplastic proliferation of follicular T helper cells.
- Clinically manifesting with a solitary nodule mostly on the head and neck area.
- Similar histological and phenotypic features are present in nodular T-cell pseudolymphoma.
- This entity cannot be distinguished with certainty from CD4+ SMT-LPD neither clinically nor histologically.
- For practical purposes, the term CD4+ SMT-LPD may be used to encompass both processes.
- This is because the prognosis and the treatment are identical.
- That is excision or radiation therapy if no regression occurs after incisional biopsy.

CUTANEOUS CD8+ AGGRESSIVE EPIDERMOTROPIC CYTOTOXIC T CELL LYMPHOMA (CD8+ AE-CTCL)

- It is an extremely rare but highly aggressive tumor.
- There is rapid evolution of necrotic and ulcerated plaques and nodules.
- Microscopically, there is a prominent epidermotropic infiltrate of mostly medium-sized CD8+ (and in 70% of the cases CD45RA positive) lymphocytes and apoptotic keratinocytes.
- Because of the poor prognosis, therapy includes multiagent chemotherapy and bone marrow transplantation.

CD8+ ACRAL T CELL LYMPHOMA (CD8+ ATCL)

- It is also a provisional entity in the revised WHO classification.
- Clinically presents with a solitary or bilateral nodule(s) at acral sites, that is, ears, face, and feet.
- Histology shows non-epidermotropic dense dermal infiltrates of small to medium-sized atypical lymphocytes.
- These cells have a characteristic immunophenotype (CD3+ CD8+ TIA-1+ CD68+).
- The clinical course is indolent with an excellent prognosis.

CUTANEOUS PERIPHERAL T CELL LYMPHOMA, NOT OTHERWISE SPECIFIED (PTL, NOS)

- It refers to those cases which cannot be classified as any of the above-mentioned entities.
- PTL, NOS cases are heterogeneous in regard to their clinical, histological and phenotypic, and prognostic features.
- The prognosis is generally poor.

CUTANEOUS GAMMA/DELTA T CELL LYMPHOMA (CGD-TCL)

- It is an extremely rare lymphoma.
- Clinically manifesting with rapidly evolving necrotic and ulcerated plaques, nodules, and larger tumors.
- Microscopically epidermotropic and/or dermal and subcutaneous infiltrates are seen.

CUTANEOUS B CELL LYMPHOMAS

Primary Cutaneous Marginal Zone/Malt Lymphoma (PCMZL)

- Skin involvement may be primary or secondary to spread from another site.
- PCMZL can arise in the setting of chronic inflammation or autoimmune diseases. For example, Borrelia burgdorferi infection has been detected by PCR.
- There is near complete absence of t (11:18).
- It is an indolent radiosensitive neoplasm.
- Clinically manifests as red-brown papules and nodules in the upper half of the body.
- **Microscopy:**
  - Dense diffuse dermal infiltrate of small lymphocytes.
  - Both plasma cells and plasmacytoid cells can be identified, some of which show Dutcher bodies.
  - Some small lymphocytes show monocytoid morphology.
  - Reactive germinal centers sometimes penetrated by neoplastic lymphocytes and lymphoepithelial lesions are seen.
- **IHC:**
  - CD20+, CD79a+, BCl 2+, BCl 6–, CD5–, CD10–, CD23–, Cyclin D1–
  - Light chain restriction is a regular feature.

Differential Diagnosis

1. Reactive lymphoid proliferation—PCMZL is favored by the following features:
   - Diffuse marginal zone cells
   - Sheets of plasma cells (some with Dutcher bodies)
   - B:T cell ratio ≥ 3:1
   - Light chain restriction
   - IHC findings.
2. Other low-grade lymphomas composed of small lymphocytes (Table 38.3).

Table 38.3

Diagnosis	*IHC findings*
Mantle cell lymphoma	CD20+, CD5+, CD23-, Cyclin D1+
CLL/SLL	CD20+, CD5+, CD23+
Follicular lymphoma	CD20+, CD5–, CD10+
Marginal zone lymphoma	CD20+, CD5–, CD10–

PRIMARY CUTANEOUS FOLLICLE CENTER LYMPHOMA (PCFCL)

- These patients present with solitary or grouped, slowly enlarging nodules mostly on the head and neck area or the upper back.
- Histologically, a follicular, diffuse, and mixed growth pattern can be observed.
- The tumor cells show a predominantly centrocytic differentiation.
- The follicles lack the normal organized germinal centers and their high rate of mitoses and apoptosis.
- **IHC:** CD20+, CD10+, CD5-, CD79a+, Cyclin D1-, BCl 2- and BCl 6+.

Differential Diagnosis

1. Reactive lymphoid infiltrate—PCFCL is favored by:
   - Lack of a polymorphous lymphocyte population
   - Loss of germinal center polarity
   - Absence of increased mitoses/apoptosis
   - CD10+, BCl 2- and BCl 6+
2. Other low-grade lymphomas composed of:
   - Small lymphocytes (Table 38.3).
   - Surgical excision and/or radiation therapy are the first-line treatment for PCFCL.
   - Clinical course is indolent.
   - Prognosis is excellent (5-year survival rate over 90%) even if cutaneous relapses occur.

DIFFUSE LARGE B CELL LYMPHOMA, LEG TYPE (DLBCL-LT)

- It is a rare but aggressive CBCL.
- Most commonly affects elderly women and clinically manifests with rapidly enlarging nodule(s) on one or both legs.
- Rarely, it may occur in other anatomic regions.
- Microscopy shows diffuse dermal growth of medium to large atypical cells.
- These cells are mainly centroblastic and/or immunoblastic.
- **IHC:** CD20+, CD10-, CD79a+, MUM-1+, bcl 2++, bcl6 +/–, IgM+, and Ki67 index is very high.

Differential Diagnosis

- Poorly differentiated squamous cell carcinoma: CK+, LCA–
- Melanoma: HMB45 +, Melan A + S-100+, LCA–
- Spread to extracutaneous organs and death because of lymphoma can occur.

EBV-POSITIVE MUCOCUTANEOUS ULCER (EBV-MCU)

- EBV-MCU is a new provisional entity.
- It is characterized by the development of a rapidly evolving solitary, sharply demarcated ulceration involving the skin, the oropharyngeal mucosa, and gastrointestinal tract.

- In these patients there is often the presence of age-related or iatrogenic immunosuppression (e.g. methotrexate and cyclosporine).
- It is an indolent process.
- There is complete remission after withdrawal of underlying immunosuppressive drugs or after radiation therapy.

POINTS TO REMEMBER

- Primary cutaneous lymphomas (CL) are non-Hodgkin lymphomas, which present in the skin without extracutaneous disease at the time of diagnosis.
- They exhibit distinct clinical, histological, immunophenotypic, and genetic features.
- It is crucial to differentiate them from systemic lymphomas as they differ in prognosis and treatment from systemic lymphomas with similar histological features.
- Thus, clinicopathologic correlation is important for diagnosis and management.
- To enable a better understanding between the various members of the patient care team it is essential that they follow the current classification schemes.

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39 Approach to the Diagnosis of Cutaneous Pseudolymphomas

Anisha Manocha

INTRODUCTION

- Cutaneous pseudolymphoma (PSL), is a group of benign lymphoproliferative conditions showing lymphocyte-rich infiltrates.
- They clinically and/or histologically simulate cutaneous lymphomas.
- Therefore, clinicopathologic correlation is essential to differentiate it from cutaneous lymphomas.
- A wide range of causative agents (e.g. Borrelia, injections, tattoos, and arthropod bites) has been described.
- Based on clinical and/or histologic presentation, four main groups of cutaneous PSL can be identified.

CUTANEOUS PSEUDOLYMPHOMA

- **T cell types:** Exhibit epidermotropism and tend to migrate into epithelial structures.
- **B cell types:** Often show a nodular structure due to their inherent tendency to form follicles.

CLASSIFICATION

- Various approaches have been used to categorize cutaneous PSL.
- For example, according to the cause, the predominating component in the lymphocytic infiltrate (T cell, B cell, or mixed) or distinct clinical features.
- In routine practice, it is not possible to recognize the cause and the phenotype at first glance without further diagnostic work-up.
- The composition of the infiltrate is determined mostly by genetic and immunologic factors of the host rather than the causative agent.
- Therefore, the same agents can in many instances induce B cell PSL (B-PSL) and T cell PSL (T-PSL) as well.
- There are four main groups of PSL based on clinical and/or histologic presentation:
  1. *Nodular PSL:* Solitary or multiple nodule(s), it resembles clinically and histologically lymphoma.
  2. *Pseudo-mycosis fungoides (pseudo-MF):* Nimics mycosis fungoides predominately on histologic grounds but clinical presentation is different.
  3. *Other PSLs:* Distinct clinical entities, for example, acral papular angiokeratoma of childhood.
  4. Intravascular PSL.

DIAGNOSTIC APPROACH

- The clinical presentation as mentioned above ranges from solitary nodule, clustered, or disseminated papules to erythroderma.
- Therefore, histological analysis plays a crucial role in the diagnostic approach.
- The important things to be noted are as follows:
  - *Infiltrate pattern:* Nodular vs epidermotropic.
  - *Size of the lymphocytes:* Mostly small, occasionally medium-sized and large cells.
  - *Immunophenotype:* T cell vs. B cell, CD4 vs. CD8, and CD30.
- Molecular studies for clonality and infectious agents, especially *Borrelia burgdorferi*, are adjunctive diagnostic tools.
- It is to be noted that mere detection of a clonal T cell or B cell population does not indicate the presence of malignant lymphoma.
- Some PSL cases have been shown to harbor clonal T cells or B cells.
- Thus, the histologic as well as the molecular findings always need to be interpreted in the correct clinical context.
- The diagnostic work-up includes the following:
  - Medical history, in particular, exposure to arthropods, allergens, and exogenic material and drugs.
  - Physical examination, including palpation of lymph nodes.
  - Examination of peripheral blood: Differential blood count and serology for infectious agents, especially *Borrelia burgdorferi,* syphilis, and HIV.
  - Staging procedures: CT or PET-CT should be considered, especially in cases of unusual manifestations.

NODULAR PSEUDOLYMPOMA (PSL)

- It represents one of the most common forms of PSL.
- Characterized by solitary or multiple nodules, simulating cutaneous T cell or B cell lymphomas on clinical and histologic grounds.
- Based on IHC, nodular PSL can be classified according to the predominant lymphocytic subset into B cell, T cell, and mixed (T cell/B cell) PSL.
- It is to be remembered that this classification is slightly arbitrary as B-PSL always contains T cells and vice versa.

CUTANEOUS B CELL PSEUDOLYMPHOMA (B-PSL)

- It is also referred to as lymphocytoma cutis or cutaneous lymphoid hyperplasia.
- **Clinical picture:** The localized form presents with a solitary nodule, measuring up to 4 cm.
- One-third of the patients develop multiple lesions (generalized form) either aggregated in clusters (agminated form) or as disseminated papules (miliarial form).
- The face, especially the nose and the cheeks; the upper trunk; and the arms are the most commonly involved sites.
- A male-to-female ratio of 3:1 has been reported.
- Approximately two-thirds of patients are under the age of 40 years and less than 10% are children and adolescents.

- **Microscopy:** There is presence of a dense nodular infiltrate, predominantly located in the reticular dermis and occasionally extending into the superficial parts of the subcutaneous tissue.
- The infiltrate is mostly composed of small lymphocytes and reactive germinal centers containing tingible body macrophages.
- The lymphocytes do not show any significant nuclear atypia.
- An admixture of diffusely scattered plasma cells is seen.
- Sometimes admixed eosinophils and a granulomatous component can be observed.
- In a majority of cases plasmacytoid dendritic cells (CD123+) are found.
- There is an admixture of a variable number of T cells, which usually account for less than 30% of the infiltrate.
- **IHC:** The majority of the infiltrate is represented by CD19+, CD20+, CD79a+, and PAX-5+ B cells.
- The cells in the reactive follicles express Bcl-6 and are negative for Bcl-2.
- The small B cells in the interfollicular area express Bcl-2 but are negative for Bcl-6.
- The networks of CD21+ follicular dendritic cells (FDCs) are sharply demarcated and regularly structured.
- Ki-67 or MIB-1 stain, the proliferative activity is elevated and mainly confined to the germinal centers.
- In the majority of cases, the expression of Ig light chains kappa and lambda by plasma cells are polyclonal (immunohistochemistry or *in situ* hybridization).

Differential Diagnosis

- **Primary cutaneous marginal zone lymphoma (PCMZL):**
  - It presents with nodular infiltrates in the reticular dermis and superficial subcutis.
  - The plasma cells in PCMZL are usually more prominent and found in sheets, especially at the periphery of the infiltrates and near the epidermis.
  - The most important histopathologic diagnostic finding is a monoclonal expression of Ig light chains (lambda or kappa) in PCMZL with a ratio of at least 5:1 or 10:1.
  - The presence and number of eosinophils is not a useful finding.
- **Primary cutaneous follicle center lymphoma (PCFCL):**
  - It shows a predominance of centrocyte-like differentiated tumor cells arranged in large neoplastic follicles.
  - Tingible body macrophages are only found in small minority of cases.
  - The neoplastic follicles show low proliferative activity in contrast with the high proliferative activity in the reactive germinal centers of B-PSL.
  - The networks of CD21+ FDCs are irregular and disrupted in contrast to the sharply demarcated and regularly structured networks in B-PSL.
  - A clonal B cell population is detectable in the majority of cases by PCR or Southern blot.
  - A vast majority of these cases do not show expression of Bcl-2 by the neoplastic centrocyte-like cells.
  - In cases of expression of Bcl-2 by the tumor cells, secondary cutaneous infiltration by a nodal FCL has to be ruled out.

- **Cutaneous infiltrates of B cell CLL/SLL:**
  - Reactive germinal centers are absent.
  - IHC: CD5+, CD23+, and CD43+.

BORRELIA-ASSOCIATED B CELL PSEUDOLYMPHOMA

- The synonyms used for this condition are lymphocytoma cutis and lymphadenosis cutis benigna.
- The diagnosis is based on histology, clinical context (history of tick bite and localization at predilection site), serologic findings, and/or detection of *Borrelia burgdorferi* species DNA in the tissue by PCR.
- Only 1% of Borrelia species infections manifest as B-PSL.
- A slight female preponderance has been observed by some authors.
- It typically affects children and occurs in early adulthood but may be seen in all age groups.
- **Clinical picture:** The most common presentation is with a solitary red to violaceous nodule.
- In 10% to 15% of the patients, multifocal skin lesions can be observed.
- Sites for predilection are earlobes, nipples, and scrotum, but the trunk and extremities may also be involved.
- **Microscopy:** A dense dermal nodular infiltrate of small B cells and reactive germinal centers is seen.
- The germinal centers tend to be larger and confluent with only a small or completely lost mantle zone.
- Lack of polarization has been reported in up to 20% of the cases.
- The histological picture resembles the neoplastic follicles in PCFCL (follicular growth pattern).
- Tingible body macrophages are found in all cases.
- Plasma cells are almost always present and found particularly at the periphery of the infiltrates.
- Eosinophils are often admixed.
- In rare cases (also known as large cell lymphocytoma) a predominance of large blasts, resembling centroblasts and immunoblasts is seen.
- These cases mimic large B cell lymphoma and are therefore prone to be misdiagnosed as diffuse large B cell lymphoma.
- **IHC:** The findings are similar to B-PSL as previously described.
- Molecular studies for the detection of *Borrelia burgdorferi* species DNA by PCR are a helpful adjunctive diagnostic tool.
- In a vast majority of the cases molecular studies show a polyclonal rearrangement of IgH genes.
- But monoclonal B cells have been observed in a few cases.
- The light chains are predominately polyclonal, but a few cases of monoclonal Ig light chains have been reported.
- **Serology:** Antibodies against *Borrelia burgdorferi* species with variable patterns are seen.
- That is, IgG and/or IgM may be elevated.
- However, serologic findings can be negative seen so negative serology does not exclude Borrelia-induced B-PSL.

PSEUDOLYMPHOMA T CELL (T-PSL) AND MIXED PSEUDOLYMPHOMA

- Nodular T-PSL is characterized by a dense dermal T cell-rich nodular infiltrate.
- It is accompanied by a variable number of B cells, which can reach up to 30% of the entire infiltrate.
- Mixed forms of PSL contain an equal number of T cells and B cells.
- All causes identified in B-PSL can also be found as underlying stimuli in T-PSL and mixed PSL.
- However, most cases are without known cause and, therefore, are referred to as idiopathic T-PSL or mixed PSL.
- **Clinical picture:** Both of them usually present with a solitary or multiple red to violaceous nodules.
- They affect patients of both genders and all age and ethnic groups.
- **Histopathology:** A dense nodular infiltrate involving the entire dermis and superficial parts of the subcutis is observed.
- The infiltrate is predominantly composed of small lymphocytes.
- A variable number of slightly enlarged lymphocytes can be seen.
- There is an admixture of a variable number of eosinophils, histiocytes, and plasma cells.
- The B cells can be arranged in small aggregates, but germinal centers are rarely found.
- Granuloma formation can be observed.
- There may be exocytosis of T-lymphocytes into the epithelia of the hair follicles, but usually, there is no significant exocytosis into the overlying epidermis.
- **IHC:** A majority of the small lymphocytes are CD4+, CD3+ T cells.
- A few activated CD30+ lymphocytes can be seen.
- The number of admixed B-lymphocytes is variable.
- Clonality studies reveal a polyclonal infiltrate in the majority of cases, but a few cases with clonal T cells have been reported.
- These have been referred to as so-called clonal PSL.
- Some of those cases may progress to overt lymphoma.

Differential Diagnosis

- Cutaneous CD4+ small/medium-sized T cell lymphoma/lymphoproliferative disorder (LPD):
  - It shows overlapping histologic and immunophenotypic features.
  - Clinically also presents with a solitary lesion located mostly on the head and neck area.
  - Clinical course is indolent.
  - It cannot be distinguished with certainty from nodular T-PSL.
  - Therefore, many authors believe that they are the same disease process.
  - The encompassing term, cutaneous CD4+ small/medium sized T cell LPD, has been introduced in the updated WHO classification.
  - The expression of PD-1 is not of any diagnostic value.
- MF in tumor stage:
  - It presents more often with medium-sized T cells with atypia.
  - Eosinophils are often admixed and, therefore, are not helpful in differentiation.
  - A monoclonal T cell receptor (TCR) rearrangement is seen.

  - The most important distinction criterion is the clinical presentation with patches and plaques preceding the tumors in MF.
- Secondary cutaneous infiltrates, for example, of angioimmunoblastic T cell lymphoma (AITL):
  - AITL shows small CD4+ and PD-1+ T cells accompanied by a significant number of B cells.
  - Clinical picture with B symptoms, serologic findings, nodal involvement, a high proliferation rate and association with Epstein-Barr virus in some of the cases of AITL are useful findings.
- Inflammatory skin disorders, lupus erythematosus (especially tumid type):
  - It can also present with dense dermal lymphocytic infiltrates.
  - Mucin deposits might be helpful.
  - Vacuolization at the interface of the epidermis and the hair follicle epithelium is not found in the tumid type of lupus erythematosus.

CD30+ T CELL PSEUDOLYMPHOMA

- They represent a histologic subtype of T cell.
- PSL of the skin.
- They are characterized by the presence of medium-sized to large atypical CD30+ T cells.
- CD30+ PSL has been reported in the context following infections and other diseases:
  - Lymphomatoid drug eruption
  - Nodular scabies
  - Hidradenitis
  - Injuries by corals
  - Viral infections
    - Orf disease
    - Milker nodule
    - Molluscum contagiosum
    - Herpesvirus infection.
- **IHC:** It shows medium-sized to large CD30+ blast-like cells.
- They are found as single units scattered throughout the infiltrate which is otherwise dominated by small T cells.
- In some cases, the underlying disease (e.g., molluscum contagiosum) can be identified.
- A significant number of B cells and plasma cells point towards a reactive process.
- It does not harbor a clonal T cell population.

Differential Diagnosis

- It includes lymphomatoid papulosis (LYP) (in particular histologic type A) and cutaneous anaplastic large cell lymphoma (ALCL).
- The CD30+ cells in CD30+ PSL are singly scattered and usually not arranged in aggregates.

PSEUDO-MYCOSIS FUNGOIDES

- Pseudo-MF is a group of disorders of varied etiology, which histologically mimic MF.
- The clinicopathologic correlation is crucial to avoid misinterpretation.

- **Microscopy:** The characteristic feature is a band-like or perivascular infiltrate of mostly small lymphocytes.
- These show exocytosis into the epidermis and may exhibit subtle nuclear atypia.
- Thus, simulating epidermotropic cutaneous T cell lymphoma.
- **IHC:** Predominantly CD4+ or CD8+ cells can be found.
- In addition, a variable expression of CD30 can be seen.
- The lymphocytes are polyclonal.
- In some diseases, such as pityriasis lichenoides et varioliformis acuta, clonal T cells are found in a significant percentage.
- However, this is to be remembered that this does not indicate malignancy or a risk for progression to lymphoma.

Differential Diagnosis

- The two main differentials are MF and Sezary syndrome.
- In cases of predominantly CD8+ infiltrate, CD8+ MF, cutaneous CD8+ aggressive epidermotropic cytotoxic T cell lymphoma, and primary cutaneous LYP (types D and E) should be considered.
- Marked nuclear atypia, predominance of medium-sized to large cells, loss of pan T cell markers, and monoclonal rearrangement of TCR genes, favor cutaneous T cell lymphoma.
- Sezary syndrome can be excluded by blood analysis.
- The individual entities that are included under Pseudo-MF are briefly summarized in Table 39.1.
- Their main differential diagnosis is MF and Sezary syndrome as mentioned in Table 39.1.

OTHER PSEUDOLYMPHOMA

Acral Pseudolymphomatous Angiokeratoma (APA)

- It was originally been described in children, but it has been shown to also affect adults.
- Some authors consider them as persistent arthropod bite reactions.
- Whereas others categorize APA as a benign vascular process with a prominent lymphocytic infiltrate, that is, a form of cutaneous PSL.
- Clinically, it manifests as a unilateral eruption of clustered red to violaceous angiomatous papules (diameter 1–5 mm) on acral sites.
- Histologically, a dense infiltrate of small lymphocytes (polyclonal T cells and B cells), eosinophils, plasma cells, histiocytes, and sometimes histiocytic giant cells is found.
- Within the infiltrate there are thick-walled vessels lined by plump endothelial cells.

ANGIOLYMPHOID HYPERPLASIA WITH EOSINOPHILIA (ALHE)

- Presently, it is considered as an angioproliferative process due to the presence of prominent, bizarrely shaped blood vessels and epithelioid endothelial cells.
- Thus, many authors use the term epithelioid hemangioma as a synonym.
- ALHE is believed to be a hyperplastic process in response to tissue damage and formation of vascular shunts.

Table 39.1: Summary of features of conditions under pseudo-MF and their differential diagnosis

	Lymphomatoid contact dermatitis	*Lymphomatoid drug reaction*	*Actinic reticuloid*	*CD8+ T cell pseudo-lymphoma in immuno-deficiency*	*Borrelia-associated T cell pseudo-lymphoma*	*Papuloerythro-derma of Ofuji*	*Mycosis fungoides*	*Sézary syndrome*
Mean age	Adults	Adults	Middle and older age	NA	60	70	55-60	Adults
Gender	Male = female	NA	Male > female	NA	Male = female	Male > female	Male > female	Male > female
Sites of predilection	Areas exposed to the allergen(s)	Variable, generalized	Face, neck	Generalized	Lower limb > trunk	Generalized, especially trunk and limbs	Initial skin lesions: Buttocks and other sun-protected areas	Generalized
Clinical picture	Eczematous and pruritic papules, patches or plaques	Rash, macularpapular eruptions	In sun-exposed areas: Persistent erythematous lichenoid papules and plaques, facies leonine	Variable: Plaques -> erythroderma, palmoplantar hyperkeratosis, lymphadenopathies	Variable: Erythema chronicum migrans, acrodermatitis chronica atrophicans, MF-like, lichenoid aspect	Itchy flat topped red to brownisch papules, erythroderma with (deck-chair sign), palmoplantar hyperkeratosis, lymphadeno-pathy	Patch, plaques and tumors (depending on the stage)	Erythroderma, palmoplantar hyperkeratosis, enlarged lymph nodes
Microscopy	Superficial band-like infiltrate, spongiosis, pseudo-Pautrier collections, eosinophils	Superficial, band like, eosinophils	Psoriasiform hyperplasia, mild spongiosis, eosinophils, coarse and vertically arranged collagen bundles in the papillary dermis	Superficial and mid-dermal infiltrate, no atypia	Band-like or deep, lichenoid aspect, lymphocytes, histiocytes (pseudorosettes), variable number of plasma cells	Pattern of chronic dermatitis with a variable epidermal hyperplasia with mild spongiosis and a mixed infla-mmatory infiltrate, predominately consist of lympho-cytes, histiocytes and eosinophils	Lining up, Pautrier collections, atypia, eosinophils are uncommon in patch MF	Often unspecific, epidermotropism maybe absent, often only mild atypia

Contd.

Table 39.1: Summary of features of conditions under pseudo-MF and their differential diagnosis

	Lymphomatoid contact dermatitis	*Lymphomatoid drug reaction*	*Actinic reticuloid*	*CD8+ T cell pseudo-lymphoma in immuno-deficiency*	*Borrelia-associated T cell pseudo-lymphoma*	*Papuloerythro-derma of Ofuji*	*Mycosis fungoides*	*Sézary syndrome*
IHC	CD4 or CD8, sometimes admixed larger CD301 cells	CD4 or CD8 predominance, admixed CD301 cells. Caveat: Loss of pan-T cell markers is possible	CD8+	CD8+, TIA-1, granzyme B	CD4+	CD4 = CD8	CD4+ or CD8+ or CD4-/CD8-, loss of pan T cell markers, admixed CD30+ cells possible	CD4+, PD-1+TOX+
Molecular diagnostics	TCR mostly polyclonal	TCR mostly polyclonal	TCR mostly polyclonal	TCR mostly polyclonal	TCR mostly polyclonal	TCR mostly polyclonal	TCR monoclonal up to 90% cases	Blood involvement
Additional findings	Identification of allergen (patch test)	None	Increased number of circulating CD81 cells in the peripheral blood, photo-sensitivity	HIV with deep immuno-suppression other type of immuno-suppression, often monoclonal cells in peripheral blood	Detection of Borrelia burgdorferi PCR, serology	Blood eosinophilia	None	Blood involvement

- Clinically, it affects both genders equally.
- Most patients are in the third or fourth decade.
- It manifests as angiomatous pink to red-brown papules or nodules most commonly found on the head and neck, especially on the face and ears.
- But can also occur at the extremities and genital area.
- Histologically, there is dermal proliferation of capillary vessels with prominent endothelial cells with typical cytoplasmic vacuoles.
- These vessels are surrounded by a dense lymphocytic infiltrate with reactive germinal centers and eosinophils.
- **IHC:** The endothelial cells are positive for CD31 and ERG but negative for podoplanin/D2-40.
- A majority of lymphocytes are of T cell lineage.
- Admixed B cells may form lymphoid follicles.
- Clonal T cells have been observed.

CUTANEOUS PLASMACYTOSIS

- It is a rare disease, which has been reported in Asian countries, especially Japan.
- It mostly affects adults.
- Clinically, characterized by multiple brownish small plaques and nodules occurring all over the body.
- Histopathology shows dermal infiltrates composed predominantly of mature polyclonal plasma cells.
- In some patients signs of a systemic involvement (e.g. lymphadenopathy, hepatosplenomegaly, hypergammaglobulinemia, increased levels of interleukin-6 in the serum, and elevated ESR) can be seen.

LYMPHOPLASMACYTIC PLAQUE (LPP)

- It is a recently described rare skin condition.
- LPP has an unknown etiology.
- The diagnosis is thus based on clinicopathologic correlation.
- Originally, it was reported in children with a predilection for the pretibial region.
- However, recent study indicates that LPP can also affect adults and involve the trunk and arms.
- A female preponderance can be observed.
- Clinically, there is a distinct presentation characterized by a longstanding plaque or circumscribed, often linear arranged reddish and brownish papules and plaques.
- Microscopy reveals a superficial, band-like, or deep nodular and interstitial infiltrate, often accentuated around adnexal structures or blood vessels.
- Epidermal hyperplasia is common.
- The infiltrate consists of lymphocytes and histiocytes with numerous polyclonal plasma cells.
- The interstitial histiocytes may form granulomas around sclerotic collagen bundles (known as pseudorosettes).
- Histiocytic giant cells and an increased number of vessels can be seen.

- LPP has to be differentiated from other conditions containing plasma cells and histiocytes as listed below:
  - Cutaneous plasmacytosis
  - Lymphocytoma cutis
  - Cutaneous marginal zone lymphoma
  - Primary and secondary cutaneous plasmacytoma
  - Infections (e.g. fungal, mycobacterial, and spirochetal).
- LPP and APA show overlapping clinical and histologic features.
- Thus, it has been hypothesized that both entities belong to the same spectrum of diseases.
- They represent a plasma cell-rich PSL with a prominent vascular component.

PSEUDOLYMPHOMATOUS FOLLICULITIS

- The term pseudolymphomatous folliculitis was first described in 1988 by Kibbi and colleagues.
- Clinically, presents with a solitary nodule preferentially located on the face.
- Histology shows a T cell predominance more often than B cells.
- In some cases, both cell types were equally distributed.
- Admixed epithelioid histiocytes and granulomas are found.
- The lymphocytic infiltrates are located throughout the entire dermis and may extend into the subcutis.
- The epidermis is spared.
- However, there is exocytosis of lymphocytes into the hair follicles.
- Hyperplasia of eccrine and apocrine ducts is often observed.
- An admixture of numerous dendritic cells with expression of CD1a and S-100 is seen in all cases.
- In one study, an unusually high number (approximately 50% of the cases) of clonal T cells and less often a monoclonal IgH rearrangement was reported.
- These cases have to be carefully differentiated from T cell or B cell lymphoma.
- Since, these can also show follicular involvement.

INTRAVASCULAR PSEUDOLYMPHOMA

- It shows benign intravascular proliferation of blasts with or without expression of CD30.
- This condition arises in areas with inflammatory skin diseases or trauma of the skin.
- It has been proposed that the obstruction of lymphatic vessels due to inflammation with disrupted immune cell trafficking may result in the accumulation of activated CD301 lymphocytes.
- These cells are large and have a blast-like morphology.
- They express T cell markers (CD3 and CD4) and in some cases CD30.
- There is no association with EBV infection.
- Clonality studies reveal the polyclonal nature of the process.
- Most important differential diagnosis is Intravascular lymphoma which is an aggressive lymphoma.
- In addition, it needs to be distinguished from intra-lymphatic histiocytosis.
- They represent a reactive proliferation of histiocytes in the lumina of lymphatics in patients with rheumatoid arthritis or orthopedic metal implants.

POINTS TO REMEMBER

- Cutaneous pseudolymphomas refer to a group of conditions characterized by lymphocyte-rich infiltrates.
- They either clinically and/or histologically resemble cutaneous lymphomas.
- A wide range of etiological agents have been described.
- They are classified into four main groups:
  1. Nodular PSL
  2. Pseudo-mycosis fungoides
  3. Other PSLs (representing distinct clinical entities)
  4. Intravascular PSL
- Clinicopathologic correlation is crucial to reach the correct final diagnosis.

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40 Diagnosis and Therapy of MALT Lymphoma

INTRODUCTION

- MALT lymphoma is also known as Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue.
- It is the third most common NHL subtype.
- Worldwide it constitutes 6–8% of all NHLs.
- In India the incidence figures as per ICMR data are as follows:
  - Indian men –2.9/100,000
  - Indian women –1.5/100,000
- The incidence is highest in Delhi, followed by Mumbai.
- Lowest incidence has been reported from Barshi which is a rural-based cancer registry.
- The Ambillikkai registry in rural Tamil Nadu has the lowest incidence of NHL in women in the world.
- According to GLOBOCAN estimates the burden of NHL in India is expected to double by 2035.

WHO Definition

- Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue is an extranodal lymphoma composed morphologically of a heterogeneous population of small B cells including:
  - Marginal zone (centrocyte-like) cells
  - Cells resembling monocytoid cells
  - Small lymphocytes
  - Scattered immunoblasts and centroblast-like cells
  - Plasma cell differentiation in a proportion of cases
- In epithelial tissues the neoplastic cells typically infiltrate the epithelium forming lymphoepithelial lesions.

Epidemiology

- MALT lymphoma comprises 6–8% of all B cell lymphomas.
- It comprises up to 50% of primary gastric lymphoma cases.
- It usually occurs in adults.
- Median age is 61 years.
- There is a slight female preponderance (M: F ratio 1:1.2).

Sites

- Most common site is the stomach
- Other sites include:
  - Small intestine.
  - Lung (14%)
  - Salivary gland (14%)
  - Ocular adnexa (12%)
  - Skin (11%)
  - Thyroid (4%)
  - Breast (4%).

Clinical Presentation

- It is clinically indolent
- Patients are often asymptomatic
- They may also present with nonspecific symptoms which vary according to the site
- It is a chronic disease with long-term surveillance and repeated biopsies
- Most MALT lymphomas arise at sites normally devoid of lymphoid tissue.
- Often there is a history of a preceding inflammatory or autoimmune condition.
- Risk factors according to the site affected are given in Table 40.1.

Table 40.1: Site-specific risk factors of MALT lymphoma

Site	*Risk factor*
Stomach	*Helicobacter pylori*
Intestine	*Campylobacter jejuni*
Orbit	*Chlamydia psittaci*
Salivary gland	Hepatitis C virus Autoimmunity (Sjögren syndrome)
Thyroid	Autoimmunity (Hashimoto's thyroiditis)
Skin	*Borrelia burgdorferi*

Microscopy

- The lymphoma cells infiltrate around reactive B cell follicles in a marginal zone distribution.
- Marginal zone B cells have the following morphological features (Fig. 40.1):
  - Small to medium-sized,
  - Irregular nuclei,
  - Moderately dispersed chromatin,
  - Inconspicuous nucleoli.
- Monocytoid appearance is seen due to accumulation of more pale-staining cytoplasm.
- Large cells resembling centroblasts or immunoblasts are usually present in minority.

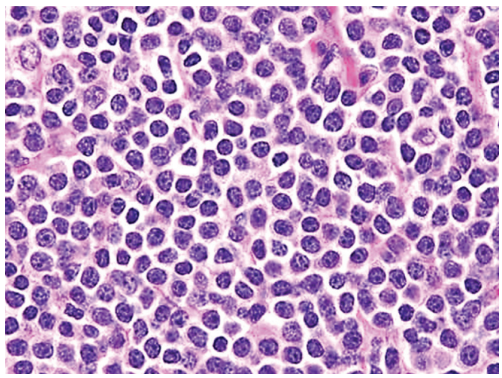

Fig. 40.1: Marginal zone B cells showing small to medium-sized cells with irregular nuclei, moderately dispersed chromatin and inconspicuous nucleoli.

- Plasmacytic differentiation (Fig. 40.2) is seen in:
  - One-third of gastric MALT lymphomas.
  - Cutaneous MALT lymphomas.
  - Thyroid MALT lymphomas.
  - Immunoproliferative small intestinal disease (IPSID) typically shows striking plasmacytic differentiation.
- Glandular tissues show lymphoepithelial lesions.
- Lymphoepithelial lesions are aggregates of three or more marginal zone cells with distortion or destruction of the epithelium, often together with eosinophilic degeneration of epithelial cells.

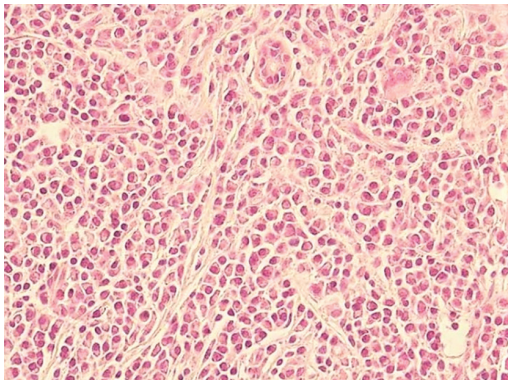

Fig. 40.2: Plasmacytic differentiation in MALT lymphoma

- If solid or sheet-like proliferations of transformed cells are present, then it should be diagnosed as diffuse large B cell lymphoma and the presence of accompanying MALT lymphoma noted.
- The term "high-grade MALT lymphoma" should not be used.
- The term "MALT lymphoma" should not be applied to a large B cell lymphoma even if it has arisen in a MALT site or is associated with lymphoepithelial lesions.

Immunohistological Features

- Positive IHC markers:
  - CD20+
  - CD 21+
  - IgM+
  - CD 43+/–
- Negative IHC markers:
  - IgD–
  - CD5–
  - CD10–
  - Bcl6–
  - Cyclin D1–
- However, it should be remembered that no specific IHC marker exists for the diagnosis of MALT lymphoma.
- Extensive, dense, diffuse infiltrate of CD20+ B cells (with or without admixed CD3 T cells) between glands and reactive follicles is highly suggestive of lymphoma.
- CK may be used to highlight lymphoepithelial lesions.

SITE-SPECIFIC MALT LYMPHOMAS

Gastric MALT Lymphoma

- Stomach is the most common site for MALT lymphoma.
- It constitutes up to 50% of the total cases.
- *H. pylori* association is seen in 85% patients.
- Clinical presentation is quite nonspecific and resembles gastritis or peptic ulcer.
- Patients may also complain of epigastric pain, nausea, or vomiting.

- Investigations also reveal nonspecific findings.
- Endoscopic findings include:
  - Ulcerations.
  - Flat mucosal changes.
  - Erosion that is spider web-shaped.
  - Multiple punched-out lesions.
  - Multiple polypoid lesions.
  - Submucosal tumor-like appearance.
- Microscopic examination shows lymphomatous infiltrate forming lymphoepithelial lesions.
- Closer examination shows neoplastic marginal zone cells and plasma cell differentiation.
- Histological scoring of gastric biopsies (Wotherspoon scoring) is shown in Table 40.2.

Table 40.2: Wotherspoon scoring system for gastric biopsies

Score	*Diagnosis*	*Histologic features*
0	Normal	No lymphoid follicles.
1	Chronic active gastritis	Small clusters of lymphocytes in lamina propria. No lymphoid follicles. No lymphoepithelial lesions.
2	Chronic active gastritis with florid lymphoid follicle formation	Prominent lymphoid follicles with a surrounding mantle zone and plasma cells. No lymphoepithelial lesions
3	Suspicious lymphoid infiltrate, probably reactive	Lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely in lamina propria and occasionally into the epithelium
4	Suspicious lymphoid infiltrate, probably lymphoma	Lymphoid follicles surrounded by marginal-zone cells that infiltrate diffusely in lamina propria and into the epithelium in small groups.
5	MALT lymphoma	Presence of dense diffuse infiltrate of marginal-zone cells in lamina propria with prominent lymphoepithelial lesions.

Immunoproliferative Small Intestinal Disease (IPSID)

- The term IPSID was adopted by WHO in 1978.
- It is a variant of MALT lymphoma that shows alpha-heavy chain production.
- Historical terms that have been used for it include:
  - Alpha heavy chain disease
  - Mediterranean lymphoma.

Clinical Presentation

- It usually occurs in older children and young adults (10–35 years).
- Patients have intermittent diarrhea.
- Colicky abdominal pain.
- Intestinal obstruction.
- Abdominal masses.
- It is associated with *Campylobacter jejuni* infection.
- The hallmark of the disease is alpha heavy chain disease seen in 20–90% cases.

Microscopy

- Lymphoplasmacytic infiltrate.
- LELs and follicular colonization may be prominent.
- It usually involves the mucosa but may extend into the submucosa.
- Mesenteric lymph node involvement is common.
- Sometimes it may transform into a large cell lymphoma.

Pulmonary MALT Lymphoma

- It constitutes 14% of all MALT lymphoma cases.
- Median age is 50–60 years.
- It is associated with Sjögren's syndrome, RA and dysgammaglobulinemia.
- Clinically patients are asymptomatic or have non-specific complaints like cough and dyspnea.
- Radiology is also nonspecific with the presence of single/multiple nodules or consolidations.

Microscopy

- A characteristic lymphangitic pattern of infiltration is seen.
- The tumor cells are seen spreading along bronchovascular bundles, interlobular septa and visceral pleura.
- They coalesce to form destructive masses that efface the pulmonary parenchyma.

MALT Lymphoma of the Salivary Glands

- It arises on a background of myoepithelial sialadenitis/benign lymphoepithelial lesion.
- Atrophic acinar tissue is infiltrated by small lymphocytes and plasma cells.
- Reactive lymphoid follicles are seen.
- Characteristically numerous epimyoepithelial islands are observed.
- The epimyoepithelial islands are solid islands of proliferated ductal epithelium infiltrated by small B lymphocytes.
- First sign is the presence of haloes or collars of pale monocytoid B cells around the epimyoepithelial islands.

Ocular MALT Lymphoma

- It was first described by Feinstein and Krause in 1952.
- Primary Ocular MALT lymphoma constitutes 1–2% of all lymphomas.
- It involves the ocular adnexa with commonly involving conjunctiva, lacrimal gland, orbit and eyelids.
- The classic "salmon red patch" appearance is seen in approximately 25% cases.
- Intraorbital mass is seen in about 75% patients.

Clinical Features

- These patients are mostly asymptomatic
- Exophthalmia (27%)
- Palpable mass (19%)
- Decreased visual acuity and ptosis (6%)
- Diplopia (2%).

Microscopy

- Epithelial tissue is absent in most POAMLs.
- It is seen involving the orbital soft tissue and eyelid.
- LELs are rare.
- There is a higher rate (40%) of plasma cell differentiation.

Thyroid MALT Lymphoma

- Primary thyroid lymphoma represents 1 to 5% of all thyroid malignancies.
- They constitute approximately 2% of all extranodal lymphomas.
- Chronic autoimmune thyroiditis (Hashimoto's disease) is a known association.
- On average, it takes 20 to 30 years to develop after the onset of lymphocytic thyroiditis.

Clinical Presentation

- Most patients present in the 7th decade of life.
- Majority of them are asymptomatic.
- They may have nonspecific compression symptoms like:
  - Dysphagia
  - Dyspnea
  - Stridor
  - Hoarseness.
- Diagnosis on FNAC is very difficult by cytomorphology alone.
- This is because the differentiation of MALT lymphomas from Hashimoto's thyroiditis is almost impossible.
- Core needle biopsy has a higher diagnostic yield.

MALT Lymphoma of Breast

- It was first reported by Lamovec in 1987.
- Mainly middle-aged and older women are affected.
- Median age of presentation is 68 years.

Clinical Presentation

- It is typically unilateral.
- Detected by physical examination or by mammography.
- Size may vary from as little < 1 cm to as large as 20 cm (median size ≈3 cm).
- Lymphoepithelial lesions (LELs) are less conspicuous.
- It is negative for rearrangements of MALT1.
- Trisomy 3, 12, and 18 have been documented in a minority of cases.
- Long-term prognosis is excellent.

MALT Lymphoma of Skin

- It constitutes 11% of all cases of MALT lymphoma.
- Median age of occurrence is 61 years.
- It comprises 2–7% of all primary cutaneous lymphomas.
- Associated with *B. burgdorferi* infection.
- Overall there is an excellent prognosis with a 5-year survival of 90–100%.

Clinical Presentation

- It presents as a solitary lesion or clusters of asymptomatic, reddish-brown to violaceous papules, nodules and plaques.
- Most commonly affected sites are back of the trunk and arms. Less common is head and neck.

Microscopy

- Nodular or diffuse infiltrate involving the reticular dermis, often extending into the subcutaneous fat.
- Grenz zone is usually present.
- LELs are rare and when present may affect the hair follicles and sweat glands.

DIAGNOSTIC CHALLENGES

- DLBCL arising in a background of MALToma.
- Follicular colonization.

DLBCL Arising in a Background of MALToma

- It is often a challenge to decide when to label a known MALT lymphoma that is undergoing transformation to DLBCL and why.
- The rate of this transformation is quite less.
- Large blasts are present in clusters (usually containing >20 large cells) or sheet-like proliferations either mixed among or separate from the small-cell component.
- WHO guidelines state that cases showing transformation to large-cell lymphoma should be diagnosed as DLBC.
- The presence of accompanying MALT lymphoma should be noted.
- The term "high-grade MALT lymphoma" is confusing and should not be used.

Follicular Colonization

- Colonization of reactive lymphoid follicles by MALToma cells creates diagnostic uncertainty.
- Selective infiltration, replacement and expansion of germinal centers may mimic follicular lymphoma.
- Immunostaining for CD21 highlights the expanded FDC networks underlying the nodules in these cases.
- Additional differentiating points are shown in Table 40.3.

Table 40.3: Differences between follicular lymphoma and follicular colonization in MALT lymphoma

Follicular lymphoma	*MALToma cells colonizing follicles*
Smaller cells with scant cytoplasm	Larger cells with abundant pale cytoplasm (monocytoid appearance)
CD 10+	CD 10–
Bcl 2+	Bcl 2–

Cytogenetics of MALT Lymphoma

- The three major translocations seen in MALT lymphomas are:
  - t(11;18)(q21;q21)/API2-MALT1
  - t(14;18)(q32;q21)/IGH-MALT1
  - t(1;14)(p22;q32)/IGH-BCL10.
- MALT lymphomas do not carry t(11;14)(q13;q32)/IGH-CYCLIN D1 or t(14;18)(q32;q21)/IGH-BCL2 translocations are typical of mantle cell lymphoma or follicular lymphoma, respectively.

t(11;18)(q21;q21)/API2-MALT1

- This translocation is the most frequently observed cytogenetic abnormality in MALT lymphoma.
- The frequency of occurrence according to the site is as follows:
  - Gastric: 25%
  - Pulmonary: 40%
  - Ocular adnexal MALT lymphomas: 5–15%
  - Rarely, in skin, salivary gland and thyroid gland.
- It is not found in other small B cell lymphomas.
- The translocation is not found in the inflammatory conditions that associate with or precede MALT lymphoma, including *H. pylori*-associated gastritis.

t(14;18)(q32;q21)/IGH-MALT1

- It is the second most frequent translocation seen in MALT lymphoma.
- Although this translocation occurs at the same chromosomal bands as the t(14;18)(q32;q21) /IGH-BCL2 found in follicular lymphoma, it is clearly distinct.
- It is found mostly in non-gastric sites.
- Ocular adnexa, lung, salivary gland and skin show frequencies varying from 5–25%.

t(1;14)(p22;q32)/IGH-BCL10

- It is seen in a small minority of MALT lymphomas.
- Stomach and lung show frequencies of 4% and 8% respectively.
- This particular translocation has not been identified in other lymphoma types.

t(3;14)(p14;q32)/IGH-FOXP1

- It is the most recently described recurrent translocation.
- It results in the overexpression of forkhead box (FOX)P1 mRNA and protein.
- The first study demonstrated its presence in 10% of the cases.
- None of the gastric, salivary gland and pulmonary MALT lymphomas studied carried the translocation.
- Clinical significance of this translocation is unclear.

Other Cytogenetic Abnormalities

- The most common is trisomy 3 (20–46%)
- Trisomy 18.

- Trisomy 12.
- Chromosomal breakpoints involving bands 1q21 and 1p34-36.

Diagnostic Work-up of MALT Lymphoma

- **Routine investigations:**
  - CBC and PS—nonspecific findings
  - LDH and β-2 microglobulin—elevated
  - Viral serology
  - *H. pylori* detection
  - BMA and biopsy, if necessary.
- **Site-specific work-up:** Gastric MALT lymphoma work-up
- **Gastroduodenal endoscopy:** Multiple biopsies from each region of the stomach, duodenum, gastrooesophageal junction and any abnormal-appearing sites are taken.
- Endoscopic ultrasound: Evaluation of invasion of the stomach wall.
- Abdominal CT scan: Regional gastric LN involvement.
- Bone marrow involvement is uncommon.
- Thus, BM biopsy for staging is not recommended for patients with negative regional lymphadenopathy.
- The work-up for non-gastric sites is summarized in Table 40.4.

Table 40.4: Site-specific work-up in non-gastric MALT lymphoma

MALT lymphorma site	*Site-specific staging procedures*
Salivary glands	Ear/nose/throat examination and ultrasound; also check for anti-SSA or anti-SSB antibodies in to investigate the association with Sjögren syndrome
Thyroid	Ultrasound ± CT scan of the neck, and thyroid function tests
Lung	Bronchoscopy with bronchoalveolar lavage
Small intestine	*Campylobacter jejuni* search in the tumor biopsy by polymerase chain reaction (PCR), immunohistochemistry, or *in situ* hybridization
Large intestine	Colonoscopy
Breast	Mammography and MRI
Ocular adnexa	MRI and ophthalmologic examination; *Chlamydophila psittaci* in the tumor biopsy and blood mononuclear cells by PCR may be considered (in endemic areas)
Skin	*Borrelia burgdorferi* in the tumor biopsy by PCR may be considered in endemic areas

Therapy for MALT Lymphoma

- **General therapy:**
  - Antibiotic therapy for infections.
  - Treatment of underlying risk factors like Sjogren's syndrome and Hashimoto's thyroiditis.
  - Radiation therapy.

- **Systemic therapy:**
  - Chemotherapy
  - Immunotherapy
  - Surgery.

Radiation Therapy

- It provides excellent disease control.
- Modest-dose (30–40 Gy) radiation is given to the stomach and perigastric nodes over 4 weeks.
- It is indicated in those patients of MALT lymphoma of the stomach who do not have any evidence of *H. pylori* infection or show persistent lymphoma after antibiotic eradication.

Systemic Therapy

- Systemic chemotherapy and/or immunotherapy with anti-CD20 monoclonal antibodies.
- Oral alkylating agents (either cyclophosphamide or chlorambucil) or purine nucleoside analog (fludarabine, cladribine) can result in a high rate of disease control.
- Anti-CD20 monoclonal antibody rituximab shows a response rate of about 70%.

POINTS TO REMEMBER

- MALT lymphoma refers to extranodal marginal zone lymphoma (MZL)
- It is the most commonly occurring subtype of marginal zone lymphoma.
- It can arise at almost any extranodal site and is often associated with chronic antigenic stimulation.
- This occurs either as a result of chronic infection (such as gastric *Helicobacter pylori* infection) or autoimmune diseases.
- MALT lymphomas may be subdivided into gastric and non-gastric tumors.
- MALT lymphomas are generally indolent and have a good prognosis with 5-year overall survival (OS) rates over 85%.
- As a result of the rarity of the disease, there are only a few randomized trials testing the various treatment options and consequently very little guidance on how to manage these patients.

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Endometrial Stromal Tumor—Recent Advances

41

Jyoti Singh

INTRODUCTION

- Endometrial stromal cells (ESCs) are derived from the following:
- Mesenchymal stem cells
- Perivascular cells
- Bone marrow-derived marrow stem cells (MSC), with subsequent development into endometrial stromal fibroblasts (hESF).

ENDOMETRIAL STROMAL TUMORS

- Comprise ~10% of uterine mesenchymal neoplasms
- 2% of all uterine neoplasms
- Endometrial stromal nodule (ESN) → 0.5% of benign tumors
  - Endometrial stromal sarcoma (ESS) → 0.2–1.5% of all malignancies
- WHO 2003 classification is shown in Fig. 41.1.

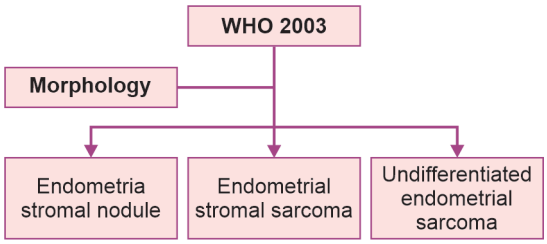

Fig. 41.1: Older classification of endometrial stromal tumors

NEW WHO 2020 CLASSIFICATION

It is as follows:

- Endometrial stromal nodule (ESN)
- Low-grade endometrial stromal sarcoma (LGESS)
- High-grade endometrial stromal sarcoma (HGESS)
- Undifferentiated uterine sarcoma (UUS)

ENDOMETRIAL STROMAL NODULE

- Age range—23 to 86 years
- Mean age—50 years
- 50% of patients are premenopausal
- Average duration: 2 months
- 10% of patients are asymptomatic
- Usually found incidentally
- Presents with abnormal uterine bleeding

- If severe may lead to anemia
- Pelvic or abdominal discomfort
- On examination
  - Uterine mass
  - Solitary, 5% have more than 2 nodules
  - Location: Intramural mass (2/3rd), polypoidal tumor (18%)

Gross

- Size: <1 cm to >20 cm
- Average size is 4–5 cm
- Well circumscribed
- Cut surface—fleshy and soft yellow to tan
- Expansile, Non-infiltrative margins
- Compresses the surrounding endometrium and myometrium
- Exception to non-infiltrative—focal finger-like projections into the adjacent myometrium that are less than 3 in number and less than 3 mm in length.

Microscopy

- Densely cellular, blue tumor (Fig. 41.2).
- Cells closely resemble proliferative-phase endometrial stromal cells.
- Mitotic activity:
  - 50%: No mitosis
  - 40%: <5 mitoses/10 hpf
  - 10%: 5–15 mitoses/10 hpf
  - Rarely: >15 mitoses/10 hpf
- Earlier there existed a category of endometrial stromal tumors with limited infiltration.
- These showed 1 mm to 1 cm stromal invasion and no vascular invasion.
- Now, under the new WHO classification they are categorized as LGESS.

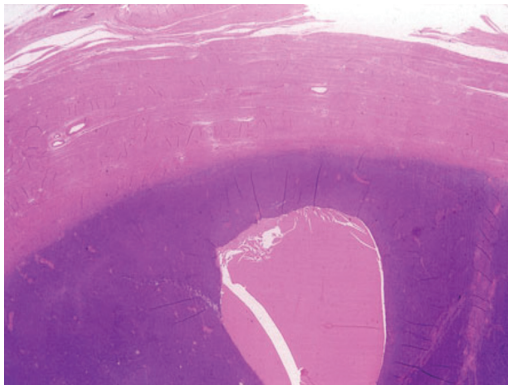

Fig. 41.2: Endometrial stromal nodule

Changes and Variants

- **Differentiation**
  - Smooth muscle
  - Skeletal muscle
  - Sex-cord stromal differentiation
- Endometrial stromal nodule with smooth muscle differentiation.
- If >30% smooth muscle differentiation in an ESN it is called mixed stromal smooth muscle tumor (earlier known as Stromomyoma).
- **Secondary changes:**
  - Cystic degeneration
  - Hyalinization
  - Calcification
  - Foamy macrophages
  - Cholesterol clefts.

Immunohistochemistry

- All endometrial stromal nodule cells are positive for CD10 (not specific).
- All endometrial stromal nodule cells are positive for ER and PR.
- Variable positivity for SMA, h-CALDESMON, etc.
- Foci of sex-cord differentiation stain with calretinin, inhibin, WT1 and CD99.

Genetics

- t(7;17):
  - Identified in over half of the cases, conventional as well as variant ESNs.
  - JAZF1-JJAZF1 (SUZ12) gene fusion.
  - The translocation generates the JAZF1-SUZ12 oncogene.
- t(6:10): Second most common.

Detection

- Conventional cytogenetics
- RT-PCR
- FISH
- At present, testing for the translocation is a research procedure
- It is not a clinical diagnostic test.

LOW GRADE ENDOMETRIAL STROMAL SARCOMA (LGESS)

- LGESS is the second most common malignant mesenchymal tumor of the uterus.
- Age range—40 to 53 years.
- 43 to 50% premenopausal.
- Location—uterine corpus.
- Extrauterine—ovary, pelvis, abdominal cavity, vulva and vagina.
- Abnormal vaginal bleeding.
- Abdominal pain.

Gross

- Uterus is typically enlarged and has an irregular contour.
- Can be polypoidal.
- Occasionally present with intra-abdominal or pulmonary metastases.
- Areas of hemorrhage, necrosis and cystic degeneration maybe seen.
- Can grow beyond the uterus.

Microscopy

- The 'tongue-like' (Fig. 41.3) pattern of myometrial and lymphovascular invasion are classical histological features that are important in the distinction from ESN.
- Cells closely resemble proliferative-phase endometrial stromal cells.

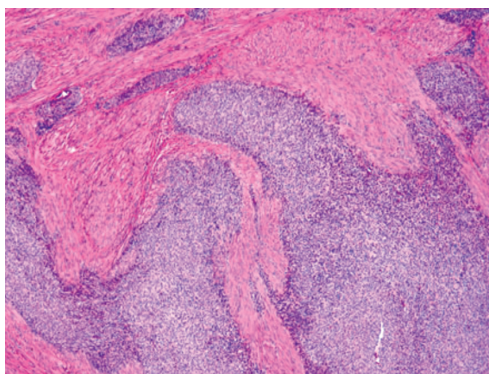

Fig. 41.3: Tongue-like pattern of myometrial invasion

- They lack significant cytological atypia
- Tumor cells show whirling around spiral arteriole-like vessels
- Mitotic activity is variable
- Typically, <3 mitoses/10 HPF
- Occasionally there are >10 mitoses/10 HPF.

Variants and Changes

- Smooth muscle differentiation
  - Typical smooth muscle cells grow as bundles.
  - A ''starburst'' like appearance.
- Rhabdoid
- Fibromyxoid
- Epithelial
  - Tubules
  - Endometrial glands
- Sex-cord
- Hyaline plaques.

Immunohistochemistry

- Similar to endometrial stromal nodule.

Genetics

- Similar to endometrial stromal nodule
  - t(7:17)
  - JAZF1-SUZ12 fusion.

Differential Diagnosis

- Cellular leiomyoma—diffuse SMA expression.
- GIST—combined use of cKit and DOG1 immunostains.

HIGH-GRADE ENDOMETRIAL STROMAL SARCOMA (HGESS)

- More aggressive subset of tumors intermediate between LGESS and UUS.
- YWHAE-NUTM2A/B (also known as YWHAE-FAM22A/B) gene fusion.
- The gene product mediates the survival of tumor cells.

Gross

- Myopermeative uterine mass
- Extrauterine tumor
- Extension/deposit
- Hemorrhage and necrosis.

Microscopy

- Tongue-like permeative pattern into the myometrium.
- Lack of perivascular whirling.

- A rich fine arborizing stromal capillary network is consistently observed resulting in a nested pattern at times.
- About half of the tumors are biphasic:
  - Low-grade areas
  - High-grade areas
- Other half shows only high-grade areas.
- Low-grade areas are either closely admixed or well-demarcated.
- Morphology is similar to LGESS.

Variant Features

- Focal sex cord-like differentiation.
- Focal pseudoglandular appearance
- Focal pseudopapillary appearance
- Focal rosette-like formation
- Importantly, the smooth muscle differentiation that is present in a subset of
- JAZF1 -LGESS has not been observed in YWHAE–NUTM2 ESSs to date.

FISH and RT-PCR

- Suggested threshold for FISH positivity of 30% of cells with rearrangements is the reference cut-off for YWHAE–NUTM2 fusion.
- A disadvantage of FISH and/or RT-PCR can be that these methods may not be routinely available.
- Recently cyclin D1 has been found to be a sensitive and specific diagnostic immunomarker.
- Currently, the term HGESS is reserved for those rare stromal sarcomas exhibiting round cell morphology.
- They harbour a t(10;17)(q22;p13) rearrangement with YWHAE-NUTM2 gene fusion.
- In the past, about 50% of these tumors may have been lumped under undifferentiated endometrial sarcoma.

Differential Diagnosis

- LGESS
- UUS
- Leiomyosarcoma- Epithelioid variant
- GIST-extrauterine sites.

UNDIFFERENTIATED UTERINE SARCOMA (UUS)

- Formerly called UES (2003 WHO)
- This wastebasket category comprises a heterogeneous group of high-grade tumors.
- They fail to fulfil the morphological and immunohistochemical criteria of translocation positive endometrial sarcomas.

Gross

- Large fleshy tumors
- Destructive infiltrative growth
- Extensive necrosis and/or hemorrhage.

Microscopy

- Sheets/fascicles of highly atypical non-descript cells that do not resemble proliferative-phase endometrial stroma (Fig. 41.4).
- Lymphovascular invasion is common
- Mitotic activity is brisk with atypical mitotic figures.

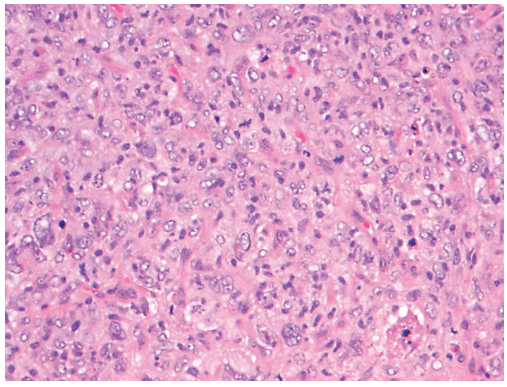

Fig. 41.4: UUS showing sheets of highly atypical cells

Genetics

- Complex karyotypes.
- TP53 mutations.
- UUS is a diagnosis of exclusion.
- Extensive sampling must be done to exclude other high-grade neoplasms.

Differential Diagnosis

- **Leiomyosarcoma:** Desmin/h-caldesmon positive, CK/EMA frequently positive.
- **Carcinosarcoma:** At least focal malignant epithelial component present.
- **Rhabdomyosarcoma:**
  - Rhabdomyoblasts
  - Desmin/myogenin/MyoD1 positive.
- **Undifferentiated endometrial carcinoma:** Discohesive cells ± low-grade endometrioid carcinoma component, at least focal keratin/EMA.
- **DLBCL:** IHC-LCA positive.

DIAGNOSTIC DIFFICULTIES

Endometrial Stromal Tumors on Endometrial Biopsies

- On biopsy or curettage, it is difficult or impossible to determine whether the stromal tumor is invasive or not.
- Thus, nonspecific diagnosis of an endometrial stromal tumor is given.
- Most endometrial stromal tumors present with large aglandular stromal fragments ≥5 mm.

POINTS TO REMEMBER

- Endometrial stromal tumors (ESTs) are a rare, and complex subset of mesenchymal uterine neoplasms.
- They have heterogeneous morphological, immunohistochemical and genetic features.
- Approximately 50% of endometrial stromal sarcomas (ESSs) occur in premenopausal women.
- The last classification of the World Health Organization (WHO) in 2020 sub-categorized ESTs into four groups: Endometrial Stromal Nodule (ESN), Low-Grade Endometrial Stromal Sarcoma (LG-ESS), High-Grade Endometrial Stromal Sarcoma (HG-ESS), and Undifferentiated Uterine Sarcoma (UUS).
- Molecular analysis of ESTs has resulted in better characterization of these tumors.

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42 GATA3 Expression in Various Cancers

WHAT IS GATA3?

- **It is a transcription factor** characterized by its ability to **bind to the DNA sequence "G-A-T-A."**
- It belongs to the **GATA family of transcription factors.**
- It was first discovered by stem cell scientists at the Princess Margaret Cancer Centre.
- **GATA family of proteins comprises 6 paralogs.**
- **GATA1, GATA2 and GATA3** have a role in hematopoiesis.
- **GATA4, GATA5 and GATA6** play a role in the development of mesoderm and endoderm-derived tissue.
- **GATA2 and GATA3** are widely expressed in many tissues
- The non-hematopoietic subfamily is composed of GATA4, GATA5 and GATA6.
- These are expressed in tissues such as intestine, heart and lungs.

COMMON FEATURES SHARED BY GATA FAMILY MEMBERS

- They contain **two transactivation domains** at the amino terminus.
- **Two zinc fingers** at the carboxyl terminus and a conserved basic region that is located immediately after each zinc finger motif.
- GATA family members bind to a consensus DNA sequence **(A/T)GATA(A/G)** in the **promoters** of genes to directly activate or repress the expression of target genes.

STRUCTURE OF GATA3

- GATA3 belongs to **the hematopoietic subfamily** of GATA zinc finger transcription factors.
- It possesses N-terminal trans-activation domains and two zinc fingers (Fig. 42.1):
  - ***C-terminal zinc finger:*** This performs DNA binding.
  - ***N-terminal zinc finger:*** It stabilizes this binding and interacts with other proteins.
- Localization: It is nuclear in location.
- GATA3 is normally expressed in many organs. These are:
  - Hematopoietic system
  - Blood vessels
  - Mammary gland
  - Bladder

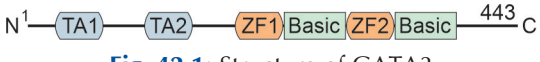

Fig. 42.1: Structure of GATA3

- Adrenal gland
- Adipocytes
- Ear
- Eye
- Seminal vesicle
- Kidney.

- **However, in many organs the expression is not seen. They include:**
  - Thyroid
  - Respiratory and pulmonary alveolar epithelia
  - Gastric and intestinal mucosae
  - Mesenchyme
  - Hepatocytes
  - Bile ducts
  - Gallbladder
  - Exocrine and endocrine pancreas
  - Testicular seminiferous tubules, Leydig cells of the testis, and ovarian stroma.

ROLE OF GATA3 IN NORMAL DEVELOPMENT

Immune System (T cells)

- GATA3 acts at multiple stages of thymocyte differentiation (Fig. 42.2)
- It is necessary for Th2 and Th9 differentiation.
- However, it has an inhibitory effect on Th1 and Th17 cell development.
- Same is true for B-lymphocytes.
- As a consequence, GATA3 overexpression is associated with enhanced allergen-induced airway inflammation and airway remodeling, including subepithelial fibrosis and smooth muscle cell hyperplasia.

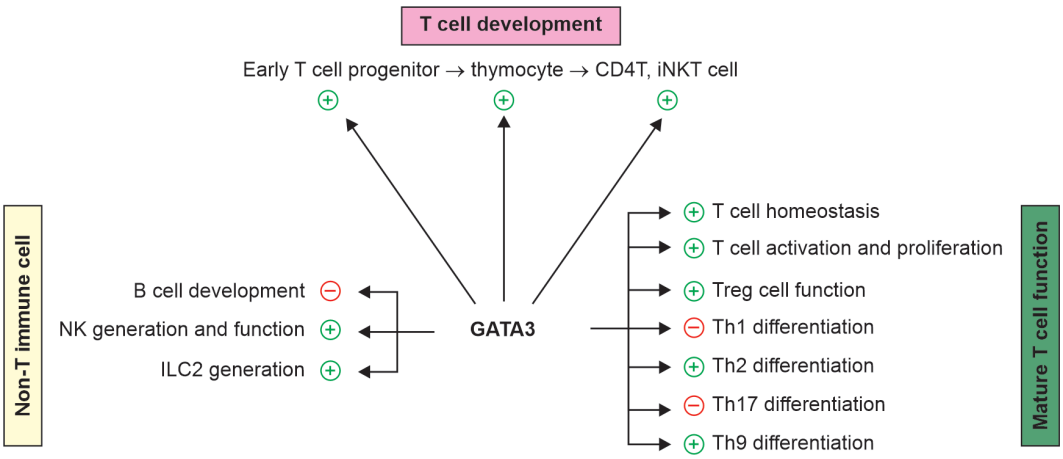

Fig. 42.2: Role of GATA3 in immune system development

MAMMARY GLAND DEVELOPMENT

- GATA3 plays an important role in the development of the breast.
- It is involved in the process of differentiation of mammary progenitor cells into luminal epithelial cells (Fig. 42.3).
- During embryonic development, it is first detected at approximately 12th day in primordial mammary bud.
- It is seen confined only to the epithelial cells.
- During puberty, it promotes ductal outgrowth.
- During pregnancy, luminal epithelial differentiation is required for lobuloalveolar development.
- GATA3 is an estrogen receptor (ER) associated marker.
- ER and GATA3 maintain a cross-regulatory positive feedback loop that result in high level of ER expression.
- GATA3 regulates FOXA1, which in turn mediates ER expression.

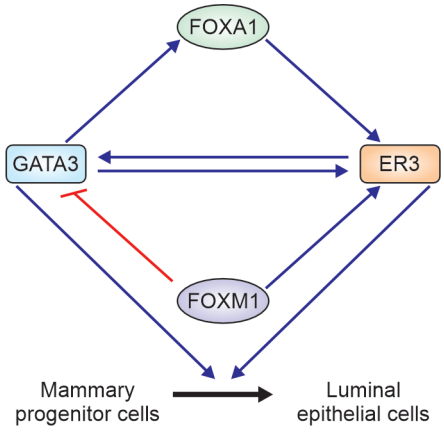

Fig. 42.3: Role of GATA3 in mammary development

- FOXM1, a forkhead transcription factor, is shown to down-regulate GATA3 expression through methylation of the GATA3 promoter.
- Therefore, **FOXM1 promotes luminal proliferation** by opposing **GATA3-mediated luminal differentiation** in the mammary gland.
- On IHC, luminal cells of ducts of a breast lobule show nuclear positivity, whereas myoepithelial cells are negative.

KIDNEY

- GATA3 is important for nephric duct elongation and development.
- Inactivation of GATA3 in the nephric duct leads to:
  - Hydronephrosis
  - Defective ureteric maturation
- GATA3 expression is seen in embryonal renal tissues within the renal tubules.
- The ureteric epithelium and some stromal lymphocytes are also positive for GATA3.

PARATHYROID

- GATA3 is critical for the differentiation and survival of parathyroid progenitor cells through regulation of **GCM2/B.**
- **Chorion-specific transcription factor GCMb** is a protein that in humans is encoded by the GCM2 gene.
- The gene product of GCM gene is thought to act as a binary switch between neuronal and glial cell determination.

SYMPATHETIC NEURONS AND ADRENAL CHROMAFFIN CELLS

- GATA3 is essential for the survival but not the differentiation of sympathetic neurons and adrenal chromaffin cells.
- It acts with HAND2 to induce noradrenergic genes during development.

EYE and EAR

- GATA3 is essential for lens cells differentiation and proper cell cycle control.
- In animal studies, it has been shown to play an important role in the morphogenesis of the mouse's inner ear.

Blood Vessels

- It plays an essential role during angiogenesis through angiopoietin 1/2 mediated signaling in large vessel endothelial cells.

Adipogenesis

- GATA3 is a negative regulator of fat differentiation.
- Therefore, it needs to be downregulated to permit expression of the peroxisome proliferator-activated receptor-gamma and preadipocyte-to-adipocyte transition.

Skin and Hair Follicle

- In keratinocytes, GATA3 is a key regulator of KLK1 expression and is involved in growth control and the maintenance of epithelial cells.
- In hair follicle morphogenesis GATA3 plays an important role in the formation of inner root sheath.
- The cells of normal skin epidermis are thus positive for GATA 3.

HDR (Barakat) Syndrome

- It is a syndrome in which the defect lies in the GATA3 gene.
- It is characterized by the following features:
  - Familial hypoparathyroidism
  - Deafness
  - Renal defects.

ROLE OF GATA3 IN NEOPLASIA

Mechanism

- It has been shown to interact with CDK4 or CDK6, and thus prevents the activation of the CDK kinases.
- It also represses p18 cell cycle regulator.
- Thus, overall it functions as a cell growth regulator that controls cell cycle G1 progression.
- It regulates the tumor cell microenvironment.
- Loss of GATA3 promotes EMT transition and tumor metastasis.

Role in Individual Tumors

- GATA3 has been thus far explored in surgical pathology as a marker for breast and urothelial carcinomas (primary and metastatic carcinomas).
- However, published data on GATA3 expression in tumors from other organs and normal tissues is limited.

Breast Carcinoma

- Since GATA3 is restricted to the luminal cells and absent in the myoepithelial cells. It has been implicated in the luminal types of Breast carcinoma.
- It has been reportedly found to be frequently expressed in breast carcinomas and is a potential marker for mammary origin of metastatic carcinoma.
- GATA3 expression seems to be more extensive in lower-grade tumors and cases with high expression may have a better survival.
- The expression is reportedly lower in triple-negative tumors.
- GATA3 interacts with **BRCA1** to repress the expression of genes associated with triple-negative and basal-like breast cancer (BLBC) including **FOXC1, p-cadherin.**
- Loss of GATA3 expression also contributes to drug resistance and aggressive BLBCs.
- Strikingly, even in cases of ER-positive breast cancers, failure to respond to hormonal therapy and poor prognosis are associated with a lack of GATA3 expression.
- Low GATA3 expression is strongly associated with the following features:
  - Higher histologic grade
  - Poor differentiation
  - Positive lymph nodes
  - ER and PR negative status.
- Lobular carcinoma is almost universally GATA3 positive.

Grading of Nuclear Labeling of GATA3 on IHC

- The tumor cells are graded as follows:
  - 0 = 0–5% of tumor cell nuclei positive
  - 1+ = 6–25%
  - 2+ = 26–50%
  - 3+ = 51–75%
  - 4+ = >75%
- Thus, <5% staining is considered as a negative score.

Prognostic Value of GATA3 in Breast Cancer

- High GATA3 expression is associated with a good prognosis in breast cancer.
- Conversely deletion of GATA3 in breast carcinoma results in dedifferentiation, decreased cell-cell adhesion and increased cell proliferation.
- Hence, more chances of tumor dissemination.

Role of GATA3 in Tumor Growth

- GATA3 expressing tumors show a moderate reduction in proliferation index.
- These tumors also harbor large areas of necrosis.
- As necrosis is likely the result of the impaired nutrient supply, it suggests GATA3 may affect tumor metabolism.

Role of GATA3 in Lung Metastases

- GATA3 plays an important role in the suppression of pulmonary metastasis in breast cancer.
- This is because of inhibitory effect on ID1/3, KRTHB1, LY6F and stimulatory effect on DLC1 and PAEP.
- Therefore, the net effect is suppression of pulmonary metastases.

UROTHELIAL NEOPLASMS

- Most urothelial carcinomas are extensively positive for GATA3.
- The exception is urothelial carcinomas with poor differentiation.
- Urothelial carcinoma frequently presents as a tumor of unknown origin.
- GATA3 may be an important marker to detect tumor metastasis from a urothelial primary.

RENAL NEOPLASMS

- In renal cell carcinomas, GATA3 is seen to be expressed most frequently in chromophobe carcinomas (~50%).
- Whereas its expression is notably rare in other carcinoma types (<2%).
- Therefore, GATA3 is of interest in subtyping renal carcinomas.
- Oncocytomas are less commonly positive (17%) but also have high numbers of positive nuclei and additionally display some cytoplasmic labeling.

SKIN TUMORS

- Strong expression of GATA3 is seen in cutaneous squamous cell carcinoma.
- High GATA3 expression in cutaneous squamous cell carcinomas parallels its expression in developing and adult epidermis.
- GATA3 is markedly less commonly detected in cervical, laryngeal, and pulmonary squamous cell carcinomas.
- Therefore, GATA3 may have some value in evaluating cutaneous versus other origin for squamous cell carcinoma.
- Basal cell carcinoma also frequently shows GATA3 expression.

OTHER EPITHELIAL NEOPLASMS

- Malignant mesothelioma (58%)
- Ductal carcinomas of the salivary gland (43%) and pancreas (37%).
- Choriocarcinomas are strongly positive for GATA3
- Yolk sac (endodermal sinus) tumor may be strongly positive
- However, it is absent in seminomas and embryonal carcinoma cells.
- Thus, it may be a useful tool for subtyping of germ cell tumors and especially highlighting trophoblastic and endodermal sinus tumor-like differentiation.

POINTS TO REMEMBER

- GATA3 is a transcription factor characterized by its ability to bind to GATA DNA sequence.
- It may be helpful in refining the diagnosis and be of prognostic and therapeutic significance in breast carcinoma.
- It is a potential marker to delineate mammary and urothelial origin of metastatic carcinoma.
- GATA3 may also be of value in the diagnosis of renal and germ cell tumors.

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Hepatocellular Adenomas: Recent Concepts

43

INTRODUCTION

- Hepatocellular adenomas (HCA) are rare, benign hepatocellular neoplasms of epithelial origin.
- They constitute approximately 2% of all liver neoplasms.
- The incidence is 3 per 1 lac per year in Europe and North America but is lesser in India.
- It is usually solitary.
- However, when >10 nodules are present against a background of normal liver it is known as ***hepatic adenomatosis.***

RISK FACTORS

- Oral contraceptives are the most important risk factor in females.
- Anabolic steroids especially in bodybuilders or given for treatment of impotence and hypogonadism.
- Type I and type III Glycogen storage diseases (GSD).
- Metabolic syndrome (men show 10 times increased risk), tobacco and alcohol abuse.
- Familial adenomatous polyposis.
- MODY3 and McCune-Albright syndrome.

Hepatocellular Adenomas and Oral Contraceptives (OCPs)

- The annual incidence of HCAs with widespread use of OCPs is approximately 3–4 women per 100000 users.
- However, there has been a fall in the incidence due to the use of OCPs containing lower doses of estrogens.
- In comparison, the annual incidence in women who have never used OCPs is nearly 1 per million.
- The natural history of HCAs closely corresponds with the dose and duration of oral contraceptives used.
- HCAs tend to regress with the withdrawal of estrogen therapy.
- However, those that do not regress show variable expression of estrogen receptors (26–73%) in these lesions.

Hepatocellular Adenomas and Glycogen Storage Disease (GSD)

- Glycogen storage disorders result in impaired glycogenesis and excessive hepatic intracellular glycogen deposits.

- They usually occur in the second decade, with a higher proportion among males (2:1).
- They are typically small, multiple and unencapsulated.
- Type I GSD:
  - Also known as von Gierke's disease.
  - It is characterized by the deficiency of glucose-6-phosphatase.
  - HAs are seen in 22–75% of cases.
  - Malignant conversion is seen in 10% of cases
- Type III GSD:
  - Also known as Cori disease.
  - It is characterized by a deficiency of glycogen debranching enzyme.
  - HAs are seen in 4.4–25% of cases.
  - Possibility of malignant conversion is minimal

CLINICAL PRESENTATION

- They are most often asymptomatic.
- Usually found as an incidental finding in patients undergoing radiological work-up for unrelated or non-specific symptoms.
- Majority of tumors are solitary (70–80%) located in the right liver lobe.
- They vary in size from 1 to 30 cm.
- Large tumors present with vague lower abdominal pain.
- Jaundice occurs due to pressure effects of the tumor on the intrahepatic biliary system.
- Raised levels of ALP and gamma-glutamyl transferase are seen in some of these cases.
- Palpable intra-abdominal mass is seen in <30% of cases. It may indicate one of the following:
  - Hemorrhage and malignant transformation (complications)
- Bleeding from ruptured HCA (risk is increased if size >7 cm) seen with subcapsular lesions, particularly in pregnancy.
- Systemic inflammatory syndrome: The inflammatory HCA (IHCA) subset may present with signs of chronic anemia or ''systemic inflammatory syndrome''.
- This includes fever, leukocytosis, abnormal liver function test results, and elevated levels of acute-phase reactants, such as CRP and SAA.

MOLECULAR PATHOLOGICAL CLASSIFICATION (2017)

- Recent advances in the understanding of the molecular-genetic pathways of oncogenesis have shown that HCAs are monoclonal neoplasms.
- They have unique molecular signatures that involve oncogenetic pathways that are distinct from HCCs.
- Based on this information, in 2017, a molecular pathological classification for HCA was proposed.
- That classification divides HA into six groups, primarily based on the molecular characteristics, as determined by immunohistochemical markers and their correlation with histologic features.
- The six subtypes are as follows:
  1. HCAs with inactivating mutations of hepatocyte nuclear factor 1α (HNF1A; HHCA).

2. β-catenin exon 3 mutated HCA (bex 3 HCA).
3. β-catenin mutated HCA exon 7/8 (bex 7, 8 HCA).
4. Inflammatory HCA (IHCA).
5. Sonic Hedgehog HCA (shHCA).
6. Unclassified HCA (UHCA).

HNF1A Inactivated HCA (HHCA)

- Biallelic inactivating mutations of hepatocyte nuclear factor 1 alpha (HNF1A) have been observed in 40 to 50% of HCA.
- HNF1A is a key transcription factor controlling several major metabolic pathways in hepatocytes.
- Hepatocytes with inactivated HNF1A show the following metabolic alterations:
  - Glycolysis activation
  - Mechanistic target of rapamycin (mTOR) pathway activation.
  - Estrogen metabolism.
  - Fatty acid synthesis deregulations with liver fatty acid binding protein (LFABP) down-expression leading to fatty acid accumulation and steatosis in tumor hepatocytes.

β-catenin Exon 3 Mutated HCA (Bex 3HCA)

- Mutations of catenin β1 (CTNNB1) gene in exon 3 (coding for β-catenin) are observed in 10 to 15% of cases.
- These mutations are canonical, leading to constitutive β-catenin activation.
- Mutations in exon 3 of CTNNB1 impair β-catenin phosphorylation, by the inhibitory complex.
- It is composed of glycogen synthase kinase 3 beta (GSK3β), APC and AXIN1.
- This prevents β-catenin degradation by the proteasome.
- CTNNB1 mutations also induce the translocation of β-catenin to the nucleus.
- There it acts as a co-transcription factor with T cell factor (TCF).
- It strongly induces the expression of WNT/β-catenin target genes, such as glutamate-ammonia ligase (GLUL).
- This subtype of HCA is associated with a higher risk of malignant transformation.

β-catenin Mutated HCA Exon 7/8 (Bex 7,8 HCA)

- This subset of HCA (5 to 10%) harbors non-canonical mutations of CTNNB1 in two hot spots in exon 7 and 8.
- These mutations are exclusive to mutations in CTNNB1 in exon.
- This mutation causes a weak activation of the WNT/β-catenin pathway.
- It is correlated with a weak overexpression of GLUL and LGR5.
- Therefore, it is also not associated with a high risk of malignant transformation.

Inflammatory HCA (IHCA)

- IHCA accounts for 35 to 45% of HCA cases.
- It is defined by the constitutive activation of the IL6/JAK/STAT pathway in tumor hepatocytes.

- This is associated with an over-expression of acute-phase inflammatory proteins like CRP and serum amyloid A (SAA).
- JAK/STAT activation is caused by activating mutations of any one oncogene involved in this pathway.
- These include IL6ST (coding for gp130, 77%), STAT3 (4%), GNAS (3%), FRK (9%) and JAK1 (1%).
- All these oncogenes induce the translocation of the STAT3 transcription factor to the nucleus.
- This leads to uncontrolled activation of the inflammatory pathway, underlining the concept of ''oncogene-induced inflammation".
- Inflammatory syndrome and anemia and/or fever are sometimes observed in these patients.
- They can be considered paraneoplastic syndromes induced by the uncontrolled production of cytokines.
- Tumor resection may suppress paraneoplastic symptoms.
- Importantly, half of bex3HCA and bex7,8HCA also show an inflammatory phenotype.
- Thus, activation of the WNT/β-catenin pathway should be assessed in IHCA.

Sonic Hedgehog HCA (shHCA)

- Recently, constitutive activation of sonic hedgehog pathway was observed in 5% of all HCAs.
- These tumors were previously considered unclassified.
- The main factor responsible for the activation of the sonic hedgehog is the somatic fusion between inhibin beta E subunit (INHBE) and GLI family zinc finger 1 (GLI1).
- This fusion gene leads to uncontrolled activation of the sonic hedgehog pathway due to the overexpression of GLI1.
- This particular subtype is associated with a higher risk of bleeding, both at the histological and clinical levels.

Unclassified HCA (UHCA)

- Less than 7% of HCA demonstrate no genetic alterations or clear-cut deregulation of signaling pathways.
- These are still considered as unclassified HCA.
- Future, work is likely to further shrink this category.

MORPHOLOGY

Gross

- HCA presents most commonly as a solitary, well-circumscribed subcapsular mass.
- It is lighter in color than the surrounding liver parenchyma, with variable hemorrhage and necrosis.
- Usually unencapsulated, subcapsular in location and 10% are pedunculated.
- Hepatic adenomatosis shows multiple variable-sized nodules (>10) in a background of normal liver.

Microscopy

- HCA is usually well-demarcated from surrounding liver parenchyma but is unencapsulated.
- It has a relatively uniform cell population resembling a normal liver, with cell plates 1–3 layers thick.
- Normal-appearing hepatocytes with abundant eosinophilic cytoplasm are seen (Fig. 43.1).
- Focally cellular atypia and nuclear pleomorphism can be seen (associated with androgen use).
- No mitoses are seen.
- HCA lacks portal zones.
- Thus, presence of bile ducts excludes this diagnosis.
- "Naked arterioles" and thin-walled blood vessels are surrounded by only a thin rim of connective tissue.
- The neoplastic hepatocytes in HCA often show cytoplasmic clearing (due to glycogen) or vacuolization (due to lipid droplets).

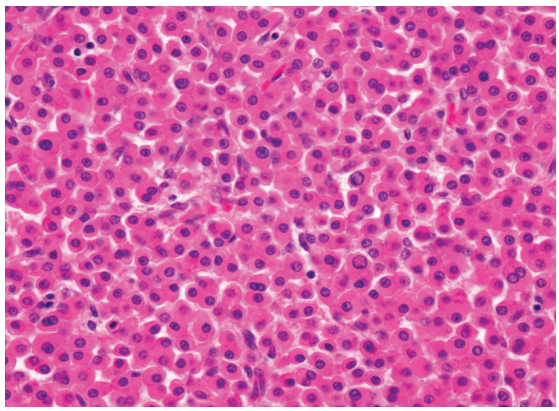

Fig. 43.1: HCA showing normal-appearing hepatocytes with abundant eosinophilic cytoplasm

Specific Morphological Findings in Various Subtypes

- In addition to the typical features of HCA, the **HHCA** subtype characteristically presents with steatosis.
- There is an absence of inflammatory infiltrate or cytologic atypia.
- The tumor cells lack immunoexpression of Liver fatty acid binding protein (L-FABP) because it is down-regulated in HHCA.
- The nonneoplastic hepatocytes surrounding the mass lesion show normal expression of L-FABP and serve as positive controls for the immunostain.
- **β-catenin exon 3 mutated HCA (b^{ex3} HCA)** shows cytologic abnormalities consisting of nuclear pleomorphism and mild atypia, multinucleation, prominent nucleoli, and/or an acinar pattern.
- Prominent cytoplasmic lipofuscin pigment can be seen.
- No steatosis or inflammation is seen.
- Glutamine synthetase (GS) is an enzyme that is involved in nitrogen metabolism.
- It is upregulated in b^{ex3} HCA because of the activation of GLUL, which is a β-catenin target gene that codes for glutamine synthetase.
- Immunohistochemically b^{ex3} HCA shows diffuse strong staining for Glutamine synthetase.
- This immunostain is negative in other HCAs.
- Like conventional HCA, telangiectatic variant of IHCA is well-circumscribed and lighter in color than the surrounding liver parenchyma.
- However, it shows prominent, dilated (telangiectatic) sinusoids filled with blood giving the cut surface a cystic, hemorrhagic appearance.
- Sinusoids within telangiectatic HCA are markedly dilated, resembling peliosis hepatis.

- Dilated sinusoids in telangiectatic HCA are often filled with hemorrhage.
- Pseudo-portal tracts with neutrophils, ductular reaction, and multiple thick-walled arterioles are seen.
- CK19 immunostain highlights the ductule-like cells in these pseudo-portal tracts.
- Serum amyloid A is positive in the tumor and negative in adjacent non-neoplastic liver.

Atypical Hepatic Adenoma

- Although rare (<10%), HCAs can transform into HCC, or contain malignant foci, or are associated with HCC.
- Certain clinical, pathological and molecular conditions are risk factors for malignant transformation.
- These are used to designate HCA as atypical.
- The presence of at least one of these criteria (Table 43.1) is sufficient for a diagnosis of atypical adenoma.

Table 43.1: Criteria for diagnosing atypical hepatic adenoma

Male sex
Female age > 50
Glycogenosis and vascular disorders
Pathological
Size of nodule > 5 cm
Focal atypia: < 5% of tumor with architectural (pseudoacini, thickened cell plates) or cytological atypia (small cell changes, nuclear atypia)
Fragmentation, and focal loss of reticulin framework
At least 1 positive marker amongst GS and HSP70
β-catenin activation, as demonstrated by immunohistochemistry (GS and β-catenin staining) or molecular biology
Absence of TERT promoter mutations

- The use of a panel of 2 immunomarkers (HSP70 and GS) has been shown to be very useful to separate atypical (60% of the cases showing at least 1 of the markers) from typical adenoma (0%).
- The combined expression of both of these markers seems also useful in the diagnosis of very well-differentiated HCC.
- As their combined expression is more often seen in HCC, as compared to atypical adenoma (38 vs. 10%)

DIFFERENTIAL DIAGNOSIS

It includes the following:
- Focal nodular hyperplasia
- Well-differentiated HCC
- Non-neoplastic liver with steatosis.

Focal Nodular Hyperplasia (FNH)

- It is a benign hepatic mass.

- It can occur in any age group. However, commonly seen in the 3rd–5th decade.
- It can be seen in both sexes (earlier studies suggested a female preponderance).
- Usually an incidental finding (80%), on surgery or autopsy.
- If clinically symptomatic, it presents as abdominal mass (15% cases).
- It is associated with OCPs, chronic alcoholics, portal hypertension and vascular malformations.
- Grossly, FNH is solitary, subcapsular with size ranging from 1-10 cm.
- It is sharply circumscribed and unencapsulated.
- On cut, FNH is tan and lighter in color.
- A central stellate scar with radiating fibrous septa dividing the mass into multiple smaller nodules is a characteristic finding.
- Nodules of mature hepatocytes are separated by a thick fibrovascular stroma.
- It has thick-walled arteries with no associated portal tracts and no accompanying bile ducts.
- The fibrovascular stroma consists of bile ductular proliferations and variable chronic inflammatory infiltrate.
- In normal liver, glutamine synthetase expression is limited to the centrilobular hepatocytes (zone 3) that directly border central veins.
- Its most important function is to aid in ammonia detoxification.
- Wnt signaling pathway has been identified as a critical regulator of zonation in the liver.
- Wnt signaling from central veins activates beta-catenin in perivenular hepatocytes, which leads to the expression of GS.
- In FNH, the centrilobular zones of β-catenin activation are expanded and this leads to an irregular, geographic (map-like) distribution of GS overexpression.
- CK7 stain highlights the strong CK7 staining in the bile ductules with focal staining in the peripheral hepatocytes of a lobule.
- It is difficult to differentiate between HCA and FNH on needle core biopsies.
- When dealing with a liver biopsy of a hepatocellular nodule in the healthy liver, the adequacy of the sampled lesion is important.
- Apart from biopsy size and thickness, the availability of representative intralesional material of the hepatocellular component of the nodule is critical.
- To address this issue, the availability of an extralesional or perilesional liver tissue is of great value.
- On routine H&E stain both FNH and HCA may have a subtle and deceptive morphology.
- Since these lesions have an increased vascular network, CD34 can help us to know their peripheral outlines.

Well-differentiated HCC

- It is an important diagnostic differential for HCA in needle biopsies.
- Histologic features in favor of HCC include thickened cell plates (often 4 cells thick), loss of the reticulin framework, cytologic atypia, mitotic figures, and pseudoacinar architecture.
- bex3HCA can also show some of these features of cytologic and nuclear atypia.
- Immunohistochemistry for glutamine synthetase shows diffuse heterogeneous cytoplasmic staining and HSP70 shows nuclear staining.

- Combined staining for both of these markers supports the diagnosis of HCC with a low sensitivity of 45%.
- Only 10% of atypical hepatocellular adenomas are positive for both of these markers.
- On the other hand, all 100% of typical HCA are negative for these markers.

Non-neoplastic Liver with Steatosis

- Non-neoplastic liver with steatosis can, at times, be nodular and cause diagnostic confusion with liver adenomatosis.
- This is particularly the case on imaging studies, because of the presence of steatosis in both entities.
- Hence, a liver biopsy should be obtained for a definitive diagnosis.
- Morphologically, the 2 conditions can look similar because unaccompanied arteries can occasionally be seen in non-neoplastic liver with steatosis.
- In such circumstances, use of IHC for L-FABP may be helpful in differentiating the 2 entities.
- A practically useful IHC panel and its interpretation in the differential diagnosis of nodular lesions of liver is shown in Table 43.2.

Table 43.2: IHC panel useful in the differential diagnosis of nodular lesions in non-cirrhotic liver

Liver mass lesion		GPC3	GS	β-catenin (Nuclear)	LFABP	SAA/CRP	CD34
HCC		+	+	–	+	–	+ (difuse)
HA	HNF1A	–	–	–	–	–	± (focal
	β-catenin HA	–	+ (strong, diffuse)	+ (nuclear)	+	–	
	Inflammatory HA	–	–	–	+	+	
	HA, NOS	–	–	–	+	–	
FNH		–	+ (map-like)	–	+	–	± (focal)
Normal liver		–	– (except a few hepatocytes in zone 3)	–	+	–	–

TREATMENT

Conservative Management

- It comprises cessation of oral contraceptives or hormonal therapy and imaging surveillance.
- The radiologic modality of choice and interval of imaging are not well-defined but MRI has been the recommended one.
- The optimal duration of follow-up is unclear and some recommend follow-up until menopause.

Radical Management

- Surgical resection remains the mainstay.
- Operative approach does not require a wide resection margin or a regional lymphadenectomy even in the case of suspected malignancy due to the minimal risk of vascular invasion or lymph node involvement.

- Laparoscopic approach may be considered for a non-hemorrhagic HCA.
- In patients with a large HCA, a two-step operative approach with portal vein occlusion may be required.
- Intrahepatic portosystemic venous shunt is a predisposing factor in the pathogenesis of HCA and its treatment requires closure by transcatheter embolization or surgical resection.
- Failure to close the shunt may result in the progression of the residual HCAs.

Transarterial Embolization (TAE)

- TAE is recommended for HCA complicated by hemorrhage.
- It is indicated within 2–3 days following tumoral hemorrhage.
- During the first week following the procedure, there may be abdominal pain, fever, right pleural effusion and elevated transaminases, which generally return to normal in the second week.
- The patient is discharged and after a month CT scan is done followed by every 3 months.
- Liver resection is only considered following resorption of the peritumoral hematoma usually six months after the initial hospitalization.
- TAE may result in regression or complete involution of the tumor.
- Absence of residual tissue on MRI may justify a non-operative approach with surveillance while radiographic persistence of HCA tissue remains an indication for surgical resection.

Radiofrequency Ablation

- Indicated in patients who are not surgical candidates or express an active desire for pregnancy with hormone-sensitive tumors.
- They should have a centrally located tumor, underlying liver disease, or demonstrate progression of residual tumors after resection.
- The size of HCA should be <4 cm for effective ablation. It is safe and well tolerated.

Liver Transplantation

- It is no longer indicated for multiple unresectable HCA tumors.
- In exceptional cases, liver transplantation may be considered for multiple unresectable HCA tumors in men or those associated with an intrahepatic portosystemic venous shunt.

POINTS TO REMEMBER

- In recent years, major advances have refined our understanding of the pathogenesis of HCAs.
- It is a heterogeneous complex disease, linking molecular subclasses with tumor phenotype and clinical behavior.
- Risk factors are linked with specific occurrences of molecular subtypes.
- Histological, immunohistochemical and imaging features are currently used to guide clinical care.
- Since HCA is a rare disease, the level of evidence derived from the literature to guide treatment is low.

- In the future, the introduction of molecular subtyping should be evaluated in prospective cohorts of patients to refine therapeutic decisions.
- The molecular mechanisms underlying HCA development and its consequence in clinical practice have been elucidated.
- Using this information pathologists must be able to identify the subtype to help guide the clinical management of patients with these tumors.

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Histopathology of Parasitic Infections of the Lung

44

INTRODUCTION

- Parasitic infections of the lung are an important but uncommonly encountered cause of pulmonary disease in most resource-rich settings.
- But frequency is much higher in poor and underdeveloped areas.
- There is a trend of increasing burden due to travel.
- Difficulty arises because they are usually diagnosed by microbiology.
- Thus, pathologists can play an important role in diagnosing parasitic infections, particularly when parasites are not considered by the clinician and the appropriate microbiology tests have not been ordered.
- A basic understanding of the parasitic infections that occur in the human lung will aid in formulating a directed differential diagnosis or provide a specific identification, which can be very helpful to direct patient care.

Types of Parasitic Infection-Associated Pulmonary Lesions

- Broadly two types of lesions can be seen in the lung—focal and diffuse.
- Important focal lesions are as mentioned below:
  - Cystic
  - Coin
  - Consolidation
  - Pleural effusion.
- Major diffuse lesions are as follows:
  - Transient pulmonary infiltrates
  - Alveolar/interstitial lung changes.

Parasite Categories that Infect Lung

- The two major categories of parasites that infect the lung are protozoa and helminths.
- Common protozoal organisms involving lungs are as given below:
  - *Entamoeba histolytica*
  - *Toxoplasma gondii*
- Common helminths involving the lung are as follows:
  - *Taenia solium*
  - *Echinococcus granulosus*
  - *Dirofilaria immitis*

- *Strongyloides stercoralis*
- Paragonimus
- Schistostoma
- *Toxocara canis*
- Wuchereria

PROTOZOA

- Lung infections caused by protozoa are usually encountered in the setting of disseminated systemic disease.
- The most common pulmonary protozoal infections include amebiasis, toxoplasmosis, and malaria lung.
- Unlike helminth infections peripheral blood eosinophilia is not seen in protozoan infections.
- Two of the most common protozoal diseases encountered by surgical pathologists are amebiasis and toxoplasmosis.

Amebiasis

- Amebiasis is primarily caused by the intestinal ameba *Entamoeba histolytica.*
- Less commonly, the free-living amebae Acanthamoeba spp. and *Balamuthia mandrillaris* may cause pulmonary disease.
- These are rarely seen in histologic sections.
- Infection with *E. histolytica* is acquired through ingestion of environmentally-resistant cysts in fecally-contaminated food or water.
- Trophozoites are then released from the cysts and colonize the colon, feeding on intestinal bacteria.
- More than 90% of infections are asymptomatic.
- The lung and pleural space are usually infected via direct extension through the diaphragm from a liver abscess.
- Thus, there is a predilection for the base of the right lung.
- Less commonly, pulmonary disease occurs through hematogenous dissemination from intestinal or hepatic infection.
- Risk factors for pleuropulmonary disease include male sex, atrial septal defect, malnutrition, alcoholism and immunocompromised status.
- Patterns of thoracic involvement include pleuritis, empyema, abscess, fistula formation (bronchohepatic or bronchobiliary fistulas), and symptoms of a mass lesion such as superior vena cava syndrome.
- These manifestations may be detected radiographically as pleural effusion, pneumothorax, mass lesion, or basal lung opacity.
- They are often associated with hepatomegaly and elevated right hemidiaphragm.
- Symptoms of pleuropulmonary disease include right upper quadrant pain, pleuritic chest pain, dyspnea, fever, cough, and hemoptysis.
- Presumptive diagnosis is usually based on detection of organism-specific IgG-class antibodies.
- Definitive diagnosis can be made by finding trophozoites in aspirated pleural fluid, abscess fluid, or sputum.
- They are usually degenerated and difficult to identify.

- Therefore, detection of *E. histolytica* DNA in tissue or aspirated fluid using PCR is very helpful for confirming the diagnosis.
- Treatment is with metronidazole or other nitroimidazole for the extraintestinal form of the disease, along with paromomycin, iodoquinol, or diloxanide furoate treatment to kill residual intestinal cysts.
- Accurate diagnosis is critical since untreated pleuropulmonary amebiasis is often fatal.
- Generally, surgical excision, biopsy, or aspiration are contraindicated for amebic abscesses due to the associated risk of rupture.
- Hence, it is only rarely observed on surgical or cytological pathology specimens.
- Grossly, amebic abscesses contain yellow-gray to red-brown opaque fluid, which is classically said to resemble "anchovy paste" or "chocolate sauce"
- In some patients, this material may be expectorated.
- In resection or biopsy specimens, trophozoites may be identified.
- Cysts are not seen.
- Microscopically, the abscesses typically contain necrotic debris which is surrounded by fibrin, granulation tissue, and mixed inflammation.
- A point to note is that neutrophils are quite rare.
- Trophozoites are often found in clusters in the wall of the lesion and can be visualized on standard hematoxylin and eosin (H&E) sections (Fig. 44.1).
- They are also highlighted using periodic acid Schiff (PAS) stain.
- Trophozoites measure 10–60 μm in greatest dimension.
- They have an abundant dense bubbly cytoplasm that may contain engulfed erythrocytes.
- The nuclei are round, slightly smaller than most host cell nuclei, eccentrically located, and have finely clumped peripherally accentuated chromatin.
- A small central karyosome (nucleolus like structure) is also seen.
- Trophozoite morphology is best appreciated in the viable organisms at the periphery of the lesion, rather than in the center of the abscess.

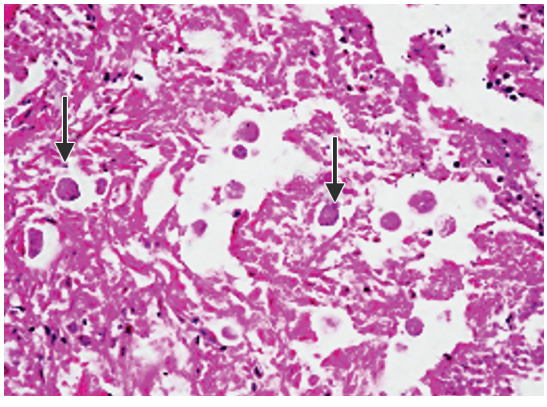

Fig. 44.1: Amebic lung abscess showing degenerated trophozoites (arrows) within the center of the lesion

Toxoplasmosis

- *Toxoplasma gondii* infection is acquired from ingestion of tissue cysts from undercooked meat, or oocysts in contaminated food or water.
- This is a common infection, involving up to half of the world's population.
- Acute *T. gondii* infection is usually asymptomatic in immunocompetent individuals.
- It may be associated with a self-limited mononucleosis-like illness, and this may rarely progress to pneumonia or myocarditis.
- Following infection, the parasite remains in the dormant state and can reactivate if immune compromise occurs.
- It can cause severe disseminated infection in immunocompromised hosts.

- The brain is the most common site of involvement in disseminated disease.
- But other organs may be involved, including the lung in 70% of cases.
- Congenital infection is another very important mode of transmission.
- The organism is transferred across the placenta to the fetus.
- It usually experiences disseminated infection in this setting, which may include pulmonary involvement.
- Toxoplasma pneumonia usually presents with cough, dyspnea and fever, with associated consolidation and mixed alveolar/interstitial infiltrates on chest CT.
- In the absence of treatment, the pneumonia is usually progressive and may be fatal.
- Diagnosis is usually established by identification of organism-specific IgG and IgM antibodies.
- However, false-positive IgM is common, and severely immunocompromised patients may have false-negative serologic tests.
- Therefore, definitive identification is through the detection of organisms or DNA in respiratory specimens.
- Treatment of choice is pyrimethamine plus sulfadoxine.
- However, this will not eradicate the dormant cysts, and ongoing prophylaxis is required to prevent relapse in immunocompromised patients.
- Grossly, areas of Toxoplasma infection are usually consolidated and hemorrhagic.
- Some areas may only be congested without consolidation.
- Microscopically, early infection is usually associated with intraalveolar fibrin, interstitial chronic inflammation, and edema.
- This progresses to diffuse alveolar damage (DAD).
- Most organisms are intracellular and if left untreated, the infection progresses to a necrotizing pneumonia, with coagulative necrosis, acute inflammation, and vasculitis.
- At this stage, parasitic organisms are usually most abundant in areas of necrosis and can be intracellular and extracellular.
- Identification of the organisms requires very careful histopathologic examination.
- They are best visualized on H&E, where the tachyzoites can be identified as round, oval, or arc-shaped organisms measuring 2–8 μm in largest size.
- They often form pseudocysts which contain bradyzoites (Fig. 44.2)
- They can be found in any cell type, but are most commonly observed in macrophages, neutrophils, and alveolar epithelial cells.
- Free organisms are often arranged in clusters without a surrounding wall (pseudocyst).
- Intravascular organisms may also be observed in overwhelming infection.
- True cysts with a well-defined wall are typically not observed in acute infection.
- They are a feature of dormant chronic infection.
- Tachyzoites do not usually stain well with GMS or PAS.

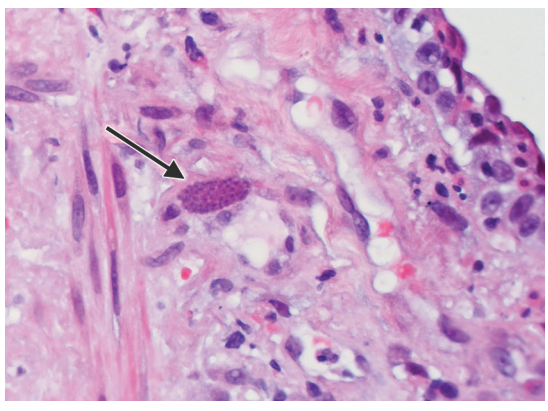

Fig. 44.2: Toxoplasma pseudocyst (arrow) containing bradyzoites

- Therefore, immunohistochemistry for *T. gondii* can be very helpful in confirming the diagnosis.
- PCR-based methods to detect organism DNA can also be performed from formalin-fixed paraffin-embedded tissue.

HELMINTHS

- Helminths are further subdivided into three subcategories: nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes).
- In contrast to protozoal infections, helminth infections are often associated with peripheral blood eosinophilia.
- The only helminth that preferentially infects the lung is Paragonimus.
- However, the lung can be infected by a variety of other helminths in the setting of systemic disease.
- These include Dirofilaria spp., Echinococcus spp., Schistosoma spp., *Strongyloides stercoralis*, *Taenia solium* (cysticercosis), Toxocara spp., and some microfilariae (associated with tropical pulmonary eosinophilia).

Trematodes

Paragonimiasis (Paragonimus Species)

- Paragonimiasis is caused by flukes in the genus Paragonimus.
- Humans become infected after ingesting raw or undercooked crustaceans (crayfish, crabs, etc.) containing the larval metacercariae form.
- *Paragonimus westermani* ("oriental lung fluke") is the most common cause of human infection worldwide.
- However, more than 10 species have been reported to infect humans.
- In the small intestine, the fluke penetrates the intestinal wall and migrates across the diaphragm into the pleura and lung.
- In the lung, the fluke matures within a cystic cavity.
- Paragonimus spp. are hermaphrodites.
- Therefore, a single fluke can produce viable eggs.
- Symptoms include cough, dyspnea, hemoptysis, chest pain, and fever.
- Pleural complications are common, including pleural effusion and pneumothorax.
- Early radiographic findings often include pleural thickening and patchy basilar infiltrates.
- Longstanding infections may resemble pneumonia, chronic pleural disease, pulmonary cysts, or nodules/masses that may calcify or cavitate.
- The masses may be hypermetabolic on PET scan, thereby mimicking a tumor.
- The diagnosis can be confirmed by identification of Paragonimus eggs in sputum, stool (if eggs are coughed up and swallowed), pleural fluid, or tissue biopsies.
- Adult flukes may be identified if the whole cystic lung lesion is excised.
- Serologic tests are also available to support the diagnosis.
- The treatment of choice is praziquantel.
- Grossly, excised areas of the lung may contain 1–3 cm cysts, which may be adjacent to or communicate with airways.
- The cysts may have a thin wall or thick wall measuring up to several millimeters in thickness.
- The adult flukes measure 7–12 mm and have a shape resembling coffee beans or lemons.

- Histologically, the lung often shows chronic eosinophilic pneumonia and granulomatous inflammation with associated eosinophils.
- Eosinophilic microabscesses and eosinophilic vasculitis are common.
- Pleural disease is commonly noted, including fibrinous pleuritis and inflammation with increased eosinophils.
- Serpiginous zones of necrosis may be present in the lung parenchyma, sometimes extending to the pleural surface.
- Acute and chronic bronchiolitis may also be present.
- In resection specimens, adult flukes may be seen within the cystic cavities, while eggs may be found in the surrounding parenchyma.
- Adult flukes can be identified by the spines present on the tegmentum, and the **ventral sucker/acetabulum.**
- Paragonimus eggs are readily identifiable on H&E and also highlighted by GMS stain.
- The eggs are large (90 mm in length).
- They have a thick yellow-brown wall which is strongly birefringent using plane-polarized light.
- A characteristic **shouldered operculum** (a flattened lid-like opening with bumps on either side is seen.
- Eggs are often associated with a foreign body giant cell (granulomatous) reaction.
- The primary differential diagnosis for large eggs in the lung is schistosomiasis. In comparison to Paragonimus eggs, Schistosoma eggs do not have an operculum.
- They have a thinner wall that usually does not display birefringence, and may have a lateral, terminal or rudimentary spine.
- The larval schistosome (miracidium) with small nuclei is usually visible within the eggs, whereas Paragonimus eggs commonly appear empty or contain undifferentiated larval structures without nuclei.

Schistosomiasis (Schistosoma Species)

- Schistosomiasis is found in the Middle East, Africa, Asia, South America and parts of the Caribbean.
- It is caused primarily by *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*.
- Humans become infected when they swim in fresh water infested with free-swimming parasites **(cercariae).**
- The cercariae can penetrate intact skin, which may lead to a maculopapular rash at the site within hours **(swimmer's itch).**
- They then migrate through the lungs and liver, to eventually reside in the blood vessels around the small intestine or bladder.
- The male and female worms, mate and the female releases eggs which migrate through the intestinal or bladder wall.
- These are then shed in the stool or urine respectively.
- Many eggs are also retained in neighboring tissues or are transported via the circulatory system to distant organs (commonly the liver via the portal blood supply).
- Several weeks after infection, patients may experience an immunologically-mediated illness with fever, cough, headache, diarrhea, and wheezing ("Katayama fever").
- It is often associated with lymphadenopathy, hepatosplenomegaly, and peripheral eosinophilia.

- Pulmonary infection is relatively rare (~10% of cases).
- This is because it occurs only when the eggs that are shed into the bloodstream are able to bypass the liver.
- This requires either primary infection of the inferior vena cava or bypass of the portal circulation.
- If the eggs reach the heart, they can travel to the lung, embedding in the walls of the pulmonary arteries and surrounding lung parenchyma.
- This may lead to diffuse opacities and multiple nodules on chest imaging.
- Over time, the chest CT pattern changes to one of miliary infiltrates and cavitary lesions.
- Coexisting liver involvement is almost always present.
- The blood flow obstruction can lead to pulmonary hypertension, with resultant shortness of breath.
- Diagnosis is usually established by identification of eggs in urine or stool.
- Serologic testing is preferred for early detection.
- The eggs can also be identified within tissue biopsies.
- Identification of eggs in respiratory specimens or lung tissue is necessary to confirm pulmonary involvement.
- Praziquantel is the treatment of choice for pulmonary schistosomiasis.
- Grossly, areas of the lung containing Schistosoma spp. eggs may be identifiable as small nodules.
- Microscopically, eggs measure 90–180 μm in greatest dimension.
- They can be identified in the lumen of small arterioles with associated granulomatous endarteritis.
- Collapsed eggs with associated granulomatous inflammation may also be identified in the surrounding lung and may be associated with increased eosinophils.
- Associated histologic changes of pulmonary hypertension are often present in advanced disease.
- These include medial hypertrophy, intimal fibroplasia, and even plexiform lesions.
- The eggs may vary in size and shape depending on the infecting species and plane of sectioning.
- They are usually collapsed due to histologic processing.
- Small parasite-derived nuclei of the Schistosoma larva (miracidium) can be seen within the egg.
- *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* eggs characteristically have lateral, terminal, and rudimentary spines, respectively.
- However, these may be difficult to identify in tissue sections.
- It is important not to mistake irregular projections from collapsed eggs as spines.
- The eggs may also show Splendore-Hoeppli phenomenon, with surrounding eosinophilic precipitate.
- The main differential diagnosis includes Paragonimus eggs, which typically have a thicker wall with strong birefringence using plane-polarized light.
- Identification of a characteristic spine and nuclei of the miracidium within the eggs supports the diagnosis of schistosomiasis.
- In contrast, Paragonimus eggs have an operculum and no spine. They generally appear empty without internal parasite-derived nuclei.

Dirofilariasis

- Dirofilaria immitis is a roundworm that causes dog heartworm disease, and sometimes accidentally infects human hosts.
- In dogs it often infects the heart.
- This is especially true in areas with numerous stray dogs or where routine deworming of domestic dogs is not done.
- The larvae cannot survive in humans, and thus humans are considered a "dead-end" host.
- Infection starts when a larva is injected via the bite of an infected mosquito and enters the bloodstream.
- The larva is unable to remain in the human heart and gets embolized to the pulmonary vasculature.
- It dies and occludes the vessel, leading to pulmonary infarction.
- Most patients are asymptomatic, and infection is discovered only when an old healed nodule is observed in imaging studies.
- Nodules typically measure 4 cm or less and may be cavitary.
- Rarely multiple nodules may be seen.
- Symptoms may include cough, chest pain and hemoptysis.
- Imaging usually shows patchy opacities with pleural effusion.
- Peripheral eosinophilia is only present in 10% of cases.
- Currently no commercially-available serologic or nucleic acid-based tests are available.
- Thus, diagnosis relies on resection of the nodule and histopathologic identification of the organism in the tissue.
- Grossly, a well-defined necrotic nodule surrounded by a normal lung is seen.
- Rarely, one or more minute (~1 mm long) thread-like tan-white worms may be identified in the area of necrosis.
- Microscopically, the worms are typically seen within a blood vessel surrounded by a well-defined region of coagulative necrosis with an outer rim of fibrosis and chronic inflammation.
- Necrotizing and non-necrotizing granulomas and increased eosinophils may also be seen.
- Eosinophilic pneumonia and eosinophilic microabscesses are rare.
- There may also be associated follicular bronchiolitis, organizing pneumonia and vasculitis, which may be rich in eosinophils, plasma cells, or giant cells.
- Overlying pleuritis is common.
- The worms are commonly seen in an advanced degenerative state, but characteristic features are usually maintained.
- They measure 100–350 μm in diameter with an externally ridged cuticle, musculature, and internal lateral ridges underlying the lateral chords.

Strongyloidiasis (Strongyloides Stercoralis)

- Strongyloides stercoralis is a roundworm endemic to the tropics and subtropics.
- Their lifecycle includes a free-living stage which allows them to survive in the environment for extended periods.
- Under the right conditions, the worms convert to the parasitic stage.

- The infectious **(filariform)** larvae can penetrate intact human skin and lead to infection.
- Irritation and a rash referred to as **"ground itch"** may occur at the site of the skin penetration.
- The larvae then migrate through the lungs, which may result in wheezing, dry cough and peripheral and localized eosinophilia (Loeffler's syndrome).
- From there, the larvae migrate up the bronchial tree and are swallowed, allowing them to reach the small intestine.
- They mature into adult females which burrow through the mucosa and lay eggs that immediately hatch to release early-stage **(rhabditiform)** larvae.
- The intestinal stage of infection may be associated with diarrhea and abdominal pain, or may be asymptomatic.
- Most larvae are shed in the stool, but a small percentage will mature into the infective filariform stage and penetrate the wall of the bowel.
- From there, they enter the bloodstream and migrate to the lungs, allowing for continuation of the life cycle.
- The resultant autoinfectious cycle allows infection to exist for decades in humans.
- The cycle is largely controlled by the immune response.
- Immunocompromised hosts are at risk for "hyperinfection syndrome" in which numerous larvae migrate from the intestine to the lungs and other organs, carrying gut bacteria with them.
- This may result in extensive intestinal mucosal damage, sepsis, and severe pulmonary manifestations including hemoptysis, dyspnea, and respiratory failure.
- Imaging can show changes in pneumonia, interstitial reticulation, or miliary nodules, and the infiltrates may be migratory, reflecting the movement of the larvae.
- Diagnosis is usually confirmed by identifying larvae in stool or sputum.
- Sensitive serologic tests are available to support diagnosis.
- But they lack specificity.
- Ivermectin is the treatment of choice.
- Grossly, areas of pulmonary infection are typically consolidated and hemorrhagic.
- Larvae may be present in the alveoli or bronchial lumens.
- Inflammatory infiltrates may range from absent to abundant mixed inflammation, with or without granulomas.
- Eosinophils may be markedly increased.
- In severe cases of hyperinfection, diffuse alveolar damage or diffuse alveolar hemorrhage may be present.
- Bacterial superinfection from intestinal flora is a common complication.
- Rarely, the organism can elicit a fibrotic granulomatous response.
- This can clinically mimic restrictive lung disease, with interlobular septal fibrosis and granulomas on histopathological examination.
- Larvae are 400–700 μm in length and 10–20 μm in diameter.
- Microscopic examination reveals rows of nuclei within the organisms which are characteristic for nematode larvae.
- The differential diagnosis for larvae in the lung includes the similar-appearing larvae of the hookworms and Ascaris lumbricoides.
- These are only rarely seen in sputum and only during their transient initial migration (Loeffler's syndrome).

- Larvae causing visceral larva migrans (Toxocara spp. and Baylisascaris procyonis) may also rarely be seen in the lung associated with eosinophilic abscesses.
- The disease typically occurs in a young child with a history of geophagia.
- The microfilariae causing tropical pulmonary eosinophilia (*Wuchereria bancrofti* and *Brugia malayi*) may occasionally be seen in the setting of acute eosinophilic alveolitis.
- This syndrome is usually seen in endemic settings (e.g. India).
- It is associated with wheezing, fever, high peripheral eosinophilia (>3000 cells/μL) along with positive titers for filarial-specific IgE and IgG.
- A history of immune compromise and concurrent gastrointestinal symptoms supports the diagnosis of strongyloidiasis.
- The notched tail of the S. stercoralis filariform larva (when seen) is also a useful differentiating feature, as it is not seen with these other larvae.

CESTODES

Hydatid Disease/Echinococcosis (Echinococcus Species)

- The most common form of pulmonary echinococcosis is caused by the dog tapeworm *Echinococcus granulosus*.
- It leads to cystic echinococcosis (hydatid disease).
- Very rarely, lung involvement can also occur due to *Echinococcus multilocularis* (alveolar echinococcosis).
- It is associated with multiple lung cysts in the setting of liver disease.
- Humans serve as an accidental intermediate host to the larval form of *E. granulosus*.
- They become infected following unintentional ingestion of eggs found in the stool of infected canids (e.g. dogs).
- Liver involvement is the most common.
- Lung is the second most common site of involvement (20–40% of patients).
- Cystic echinococcosis typically presents as a slowly growing cystic lesion.
- Small unruptured cysts are generally asymptomatic, and eosinophilia is uncommon unless there is cyst rupture.
- As the cyst increases in size, it can cause compressive symptoms.
- When it ruptures into an airway, patients may present with abrupt onset of shortness of breath, chest pain, fever, cough, hemoptysis, and expectoration of salty-tasting fluid or material resembling grape skins.
- Rupture into the pleural space can cause empyema or hydropneumothorax.
- Radiographically, the cysts are usually solitary with surrounding fibrous capsule and compressed lung.
- They range in size from 1 to 20 cm.
- Sometimes smaller "daughter cysts" are observed within the larger cyst.
- Air-fluid levels are a sign of rupture.
- The "water lily sign" is considered pathognomonic for echinococcosis.
- It occurs when the inner lining of the cyst detaches and floats on the remaining fluid.
- Following cyst rupture, the release of parasitic antigens into the systemic circulation may lead to allergic symptoms including itching, urticaria, hypotension and even anaphylaxis.
- Diagnosis is usually based on clinicoradiographic features in conjunction with positive serology.

- Cytological preparations from aspirated fluid or expectorated sputum may be examined for immature tapeworms or free hooklets.
- Biopsy may be performed if the diagnosis is not suspected.
- Surgical removal is indicated if there is impending rupture.
- Treatment usually includes albendazole therapy in conjunction with surgical resection of the cyst.
- The cyst may first be injected with a scolicidal agent such as hypertonic saline to kill the mitotically-active cyst lining, so as to minimize the consequences of accidental intraoperative rupture.
- It is commonly performed as a component of the PAIR procedure (puncture, aspiration, injection and reaspiration).
- Grossly, the cyst has a fibrous capsule, measuring up to 3 mm in thickness, and surrounded by compressed host tissue.
- The fluid often has a fine gritty consistency due to the presence of the immature hooked tapeworms and free hooklets in the fluid ("hydatid sand").
- Microscopically, cyst wall is composed of an outer lamellated acellular eosinophilic material (Fig. 44.3).
- Just below the cyst wall is the germinal layer from which the immature tapeworms (protoscoleces) arise.
- Protoscoleces are ovoid and measure approximately 100 μm in diameter.
- They have two internal circular rows of hooklets and four cup-shaped suckers.
- Free hooklets are released from degenerating protoscoleces.
- They are 15–30 μm in length, refractile and weakly birefringent.
- All larval cestodes contain small variably sized calcified bodies within the stroma of the organism (calcareous corpuscles).
- If the cyst has ruptured, there is typically associated granulomatous and acute inflammation.
- In long-standing lesions, the hooklets and germinal layer may become quite degenerated and difficult to recognize.
- Polarization or viewing with a narrowed condenser may aid in identifying rare hooklets in the background of fibrosis and granulomatous inflammation.
- The lamellated outer layer can also be highlighted by GMS and PAS stains (which are negative in simple acellular fibrin).
- The hooklets stain dark red with Ziehl-Neelsen.
- The alveolar form of echinococcosis shows tumefactive growth of cysts, which diffusely infiltrate the host tissue.
- There is no surrounding lamellated layer, and protoscoleces are rarely produced.

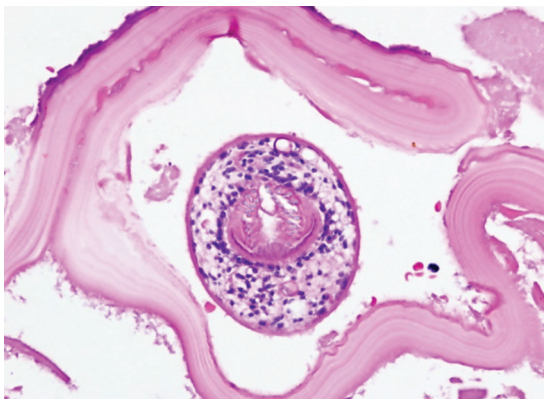

Fig. 44.3: Scolex surrounded by laminated membrane lining the hydatid cyst

Cysticercosis (Taenia solium)

- Taenia solium, commonly known as the 'pork tapeworm', infects humans either in the adult or larval stage.

- When humans ingest the larval form (cysticerci) in undercooked pork, they serve as the definitive host.
- The adult tapeworm lives in the intestinal tract.
- This type of disease is relatively benign and only occasionally causes symptoms.
- More worrying is when humans ingest eggs that are shed in the stool of a human with the intestinal tapeworm infection.
- The eggs hatch following ingestion and enter the bloodstream.
- They disseminate throughout the body to form cysts in various organs, including the skin, eyes, brain, lungs and muscles.
- This is called cysticercosis and humans serve as the intermediate host.
- The symptoms depend on the location of the cysts.
- Thoracic involvement usually includes only the respiratory muscles.
- Pleuropulmonary parenchyma may rarely be involved.
- Over time, the lung cysts calcify and may be seen on chest X-ray as radiolucent nodules measuring 3–10 mm in maximum dimension.
- In some cases, a scolex can be identified within fluid-filled nodules on chest CT.
- Diagnosis is usually made using serology.
- Cross-reactivity with antibodies to Echinococcus spp should be kept in mind.
- The organisms can also be identified in histologic sections.
- Albendazole is the treatment of choice.
- Grossly, cysticerci have a thin wall and are filled with clear fluid.
- Microscopically, an intact cysticercus can be identified by the presence of a single invaginated scolex with four suckers and refractive hooklets.
- The scolex is attached at an infolded neck region.
- The immature scolex within a cysticercus has a similar appearance to the protoscoleces of *E. granulosus.*
- The scolex is single and attached to a characteristic neck region.
- Protoscoleces are smaller, usually numerous, and often seen floating 'free' within a cyst.
- Cysticerci are smaller than echinococcal cyst and are not surrounded by a laminated layer.
- Intact viable cysticerci are not associated with an inflammatory response (Fig. 44.4).
- Death of the organism with degeneration leads to intense host tissue inflammation, including neutrophils, eosinophils, and macrophages.
- In this setting, despite difficulty in identification the neck region of the cysticercus can be seen, along with occasional suckers or hooklets.
- Calcareous corpuscles can also aid in supporting the diagnosis of a cestode infection, since they are present in all cestodes (adult and larval forms).
- If the cysticerci are degenerated, diagnosis can be particularly difficult.
- Correlation with serologic testing and clinicoradiographic features are helpful in this regard.

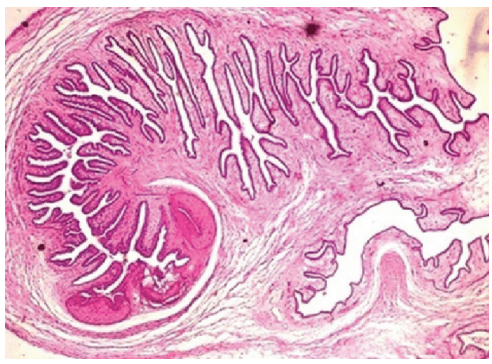

Fig. 44.4: Cysticercosis larva surrounded by a thick fibrous wall

Parasitic Mimics

- Several things mimic parasites.
- Aspirated foreign material is probably the most commonly observed parasite mimic.
- Vegetable matter such as small seeds may mimic parasitic eggs due to their thick cell walls.
- Nuts, larger seeds and lentils can also be mistaken for cross-sections of parasitic organisms.
- This is due to internal structures that may mimic parasitic organs.
- Vegetable matter can be recognized by the characteristic appearance of several compartments within a prominent thick and rigid cell wall.
- Aspirated foreign material may also be polarizable.
- The characteristic bronchiolocentric distribution of aspiration is also a clue to the correct diagnosis.
- Other mimics of parasites in cytology specimens include detached ciliary tufts (ciliocytophthoria) or degenerated ciliated epithelial cells, which may be mistaken for ciliated or flagellated parasites.
- However, these detached ciliary tufts are very small and are generally arranged in a tangle still attached to the terminal bar.
- Curschmann spirals are typically seen in inflammatory conditions with abundant eosinophils.
- They may be mistaken for parasites especially helminths.
- However, they can be differentiated by their spiraled configuration, lack of internal structures, and variability in size.

POINTS TO REMEMBER

- Parasitic infection can be a diagnostic challenge for histopathologists.
- This is due to the lack of awareness.
- A basic knowledge of the differential diagnosis of the organisms that can infect the lung, along with their typical histologic features, can aid in definitive identification.
- This is especially true when clinical and radiographic information is also taken into account.
- Many clinicians are not familiar with parasites that infect the lung.
- Thus, pathologists may play an important role by providing valuable information in their final reports.

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HIV-Associated Renal Diseases

45

INTRODUCTION

- Association between HIV and renal disease was first reported in 1984 in New York City and Miami.
- HIV-seropositive patient developed a renal syndrome progressive renal failure and proteinuria.
- This condition was given the name of HIV-associated nephropathy (HIVAN).
- The most common biopsy finding was focal segmental glomerulosclerosis (FSGS).

EPIDEMIOLOGY

- Incidence of end-stage renal disease (ESRD) due to HIVAN has increased more rapidly than other etiology of renal diseases.
- It is now the 3rd leading cause of ESRD.
- Prevalence is 3.5–48%.
- In India it is 3.5–7%.
- Both HIV and HAART therapy (due to ART nephrotoxicity) are implicated in the pathogenesis of HIVAN.
- The global as well as trends from India indicate that the incidence and prevalence of HIVAN are increasing every year.

RACIAL PREDILECTION IN HIVAN

- A marked racial predilection was seen in black and Hispanic patients.
- Some authors suggest that genetic factors are important determinants of HIVAN pathogenesis.
- The Duffy antigen/receptor for chemokines (DARC) has been proposed as a candidate gene involved in HIVAN pathogenesis.
- The DARC promoter has a high prevalence of polymorphisms in black patients.
- Another investigator has demonstrated increased DARC expression in renal specimens from children with HIVAN.

SPECTRUM OF HIV-ASSOCIATED RENAL DISEASES

It includes the following entities:

- Acute renal failure: It manifests with hypovolemia, hypotension and hypoalbuminemia.
- ART nephrotoxicity

- Chronic HIV nephropathies—they can be either:
  - Glomerular-dominant or
  - Vascular-dominant.

KDIGO

- In March 2017, Kidney Disease: Improving Global Outcomes (KDIGO) convened a multidisciplinary, international panel of clinical and scientific experts.
- The aim of this meeting was to identify and discuss key issues relevant to the optimal diagnosis and management of kidney disease in HIV-positive individuals.
- The primary goals were to define:
  a. Pathology of kidney disease in the setting of HIV-infection.
  b. Pathologic classification of HIV-related kidney diseases.
  c. Role of genetics in the natural history, diagnosis, and treatment of kidney disease in HIV-positive individuals.
  d. The renal risk-benefit of ART.
  e. Treatment of end-stage kidney disease (ESKD) in HIV-positive individuals.

PATHOLOGIC CLASSIFICATION OF HIV-RELATED KIDNEY DISEASES (KDIGO)

- Glomerular-dominant
  a. Podocytopathies (all characterized by extensive foot process effacement)
    - Classic HIVAN
    - FSGS (NOS) in the setting of HIV
    - Minimal change disease in the setting of HIV
  b. Immune complex-mediated glomerular disease
    - IgA nephropathy in the setting of HIV
    - Lupus-like glomerulonephritis in the setting of HIV
    - Lupus nephritis in the setting of HIV
    - Membranous nephropathy in the setting of HIV
    - Membranoproliferative pattern glomerulonephritis in the setting of HIV
- Tubulointerstitial-dominant
  a. Tubulointerstitial injury in the setting of classic HIVAN
    - Hyaline droplet tubulopathy
    - Tubular microcysts
    - Tubulointerstitial inflammation
  b. Acute tubular injury or acute tubular necrosis
    - Ischemic
    - Toxic (associated with ART vs. other)
  c. Drug-induced tubulointerstitial nephritis (other than ART)
    - Antibiotics
    - Proton pump inhibitors
    - NSAIDs
    - Other
  d. Direct renal parenchymal infection by pathogens (bacterial, viral, fungal, protozoal, etc.)
  e. Immunologic dysfunction-related tubulointerstitial inflammation
    - Diffuse infiltrative lymphocytosis syndrome (DILS)
    - Immune reconstitution inflammatory syndrome (IRIS)

- Vascular-dominant
  - Thrombotic microangiopathy in the setting of HIV
  - Arteriosclerosis
- Other, in the setting of HIV infection
  - Diabetic nephropathy
  - Age-related nephrosclerosis.

PATHOGENESIS

- Most commonly infected cells in HIV infection are **renal epithelial cells.**
- They act as an important reservoir for HIV.
- It has been reported that despite an undetectable viral load in the peripheral blood while on HAART, the patients continued to express HIV in renal epithelial cells as determined by RNA *in situ* hybridization.
- Thus, even in the face of an optimal virologic response to antiretroviral therapy and clinical remission of HIVAN.
- HIV infection persists in the renal epithelium and the virus remains transcriptionally active at a low level.
- **HIV-transgene** is expressed in renal glomerular and tubular epithelial cells.
- This transgene expression in renal epithelial cells is required for the development of the HIVAN phenotype.
- The role of direct infection of renal parenchymal cells in HIVAN in a macaque model of HIV-induced renal disease has been shown.
- CD4, receptor for HIV and CCR5 and CXCR4, the major co-receptors for HIV are not expressed in most renal epithelial cells.
- Thus, the mechanism by which HIV gains entry into renal epithelial cells is unknown.
- Apoptosis, or programmed cell death, has been implicated in the pathophysiology of HIV-related renal diseases.
- The effect appears to be mediated by tumor necrosis factor TNF alpha and caspase activation.
- The fibrogenic cytokine **TGF-beta** has been implicated in the pathogenesis, it induces extracellular matrix deposition, including stimulation of matrix synthesis and inhibition of matrix degradation.
- Increased IFN-alpha levels in renal glomerular and interstitial tissue predispose to the development of fibrosis and sclerosis.

PODOCYTOPATHIES

- They are characterized by extensive podocyte foot process effacement.
- There is absent or minimal immune complex deposition.
- Proteinuria is seen.
- The causal relationship between HIVAN and HIV infection is now well-established.
- It is mediated by the following:
  - Direct HIV infection of renal epithelial cells.
  - Intrarenal viral gene expression.
  - Dysregulation of host genes governing cell differentiation and cell cycle.
- The role of genetic susceptibility has also been suggested.

Classic HIVAN

- Classic HIVAN is defined as collapsing glomerulopathy and attendant tubulointerstitial disease, including tubular microcyst formation, interstitial inflammation and tubular injury.
- Glomerular "collapse" is defined as at least 1 glomerulus with collapse of glomerular basement membranes.
- This is accompanied by hypertrophy and hyperplasia of the overlying glomerular epithelial cells.
- These hyperplastic cells may fill the urinary space, forming pseudocrescents.
- The proliferating podocytes fill the Bowman's space caused by collapsed tufts and result in the formation of so-called pseudo-crescents.
- These differ from the true crescents of Crescentic GN by the following features:
  a. Centripetal location,
  b. Presence of a cleft-like space between the pseudo-crescents and the parietal epithelium,
  c. Epithelioid appearance of cells forming the pseudo-crescents, and
  d. Absence of fibrin in the pseudo-crescents.
- The tubulointerstitium shows focal tubular microcysts containing glassy casts.
- This is associated with tubular atrophy, interstitial fibrosis, and inflammation.
- Tubular microcysts are easily distinguished from tubular thyroidization based on their larger diameter, irregular size and the absence of tubular atrophy or colloid-type casts.
- In late stages, the sclerotic tuft is retracted into a tight solid sphere, capped by a monolayer of cobblestone epithelium.
- This has been described as resembling a "fetal glomerulus".
- Immunofluorescence demonstrates deposits of immunoglobulins (IgA, IgM, IgG) and complement (C3, C4, C1q) within the mesangium.
- Electron microscopy shows effacement of foot processes.
- The glomerular endothelial cells may contain intracytoplasmic tubuloreticular inclusions.

Diagnostic Features

- HIV-associated nephropathy (HIVAN), formerly known as AIDS-associated nephropathy, is characterized by the following findings:
  - Nephrotic range proteinuria >3.5 gm/day
  - Azotemia
  - Normal to large kidney on ultrasonographic images
  - Normal pressure
  - Focal segmental glomerulosclerosis (FSGS) on biopsy findings.

FSGS (NOS)

- In ART-treated patients, noncollapsing FSGS (NOS) is more commonly encountered.
- Causality is presumed when no other etiology for FSGS can be demonstrated.
- Viral load is often undetectable and biopsy findings may be difficult to distinguish from arterionephrosclerosis of hypertension, aging, and APOL1-associated nephropathy.

- Such cases typically lack prominent tubulointerstitial disease, and the degree of podocyte effacement is generally less severe than in HIVAN.
- These differences have been hypothesized to reflect attenuation of the renal phenotype by ART.
- Microscopically discrete segmental scars with segmental adhesions to Bowman's capsule are seen.
- No collapsing features or glomerular epithelial cell hyperplasia are identified.

Podocytopathy in Perinatal HIV Infection

- In addition to classic HIVAN, children with perinatal HIV infection can present with minimal change disease or diffuse mesangial hypercellularity.
- Numerous endothelial tubuloreticular inclusions and marked foot process effacement are also seen.
- Tubular microcysts and interstitial inflammation are often lacking.
- However, these cases are rare in the ART era.

IMMUNE COMPLEX-MEDIATED GLOMERULAR DISEASE

- Numerous forms of immune complex-mediated glomerular disease have been reported in HIV-positive individuals.
- It was recommended at KDIGO that the commonly used term "HIV immune complex kidney disease" (HIVICK) should be replaced with a specific description of the pattern of immune complex disease "in the setting of HIV."
- A unique lupus-like nephritis with full-house immune staining but negative serologies and no clinical signs of systemic lupus erythematosus have been reported in HIV-positive individuals.
- However, true lupus nephritis also occurs.
- It remains unclear whether IgA nephropathy in the setting of HIV is coincidental and related to undergalactosylated IgA1 or due to deposition of IgA directed to viral antigen.
- An unusual ultrastructural appearance of subepithelial deposits, or "ball in cup" lesion has been described.
- Other secondary causes should be sought in all such cases.

TUBULOINTERSTITIAL DISEASE IN THE SETTING OF HIV

- Acute tubular necrosis may occur in association with sepsis, volume depletion, and other ischemic or toxic insults.
- The commonly used antiretroviral agent tenofovir disoproxil fumarate can cause proximal tubulopathy with characteristic dysmorphic mitochondria.
- Acute tenofovir nephrotoxicity is characterized by irregular proximal tubular profiles with atypical, irregular lining epithelial cells and mild interstitial edema.
- The atypical proximal tubular cells display loss of brush border, marked irregularity of tubular epithelial height and shape, focal shedding of cytoplasmic fragments, and enlarged atypical nuclei with prominent nucleoli.
- **Chronic nephrotoxicity** shows interstitial fibrosis and mild inflammation with focal tubular atrophy.

- The tubules show focal loss and flattening of lining epithelium leaving some desquamated tubular basement membranes, as well as prominent epithelial simplification and irregularity with atypical nuclei.
- Tubulointerstitial nephritis can also occur secondary to antibiotics, proton pump inhibitors, nonsteroidal anti-inflammatory drugs, protease inhibitors, and other medications, as well as in response to mycobacterial infection.
- Direct infection of the renal parenchyma by other pathogens can also occur.
- However, two rare but distinct forms of tubulointerstitial injury that relate to immunologic dysfunction in the setting of HIV infection merit attention.
- **Diffuse infiltrative lymphocytosis syndrome (DILS)** is a hyperimmune reaction against HIV that involves the kidneys in approximately 10% of cases.
- **Immune reconstitution inflammatory syndrome (IRIS)** is an inflammatory disorder associated with paradoxical unmasking or worsening of preexisting infectious processes after ART initiation.
- It rarely involves the kidney.
- Both conditions are characterized by prominent CD8 T cell infiltrates.

VASCULAR-DOMINANT DISEASES IN THE SETTING OF HIV

- Thrombotic microangiopathy (TTP/HUS) was reported in the early years of the AIDS epidemic but is rare in the ART era.
- A role for direct endothelial dysregulation by HIV has been proposed.
- The pathogenesis of HIV-associated TTP/HUS is unknown.
- The disease is thought to be a result of **endothelial cell injury and platelet deposition** within the microvasculature.
- Coexisting infections, cytotoxins, deficiencies of inhibitors of platelet aggregation and coagulation cascade abnormalities have all been implicated.
- It has been suggested that **cytotoxic HIV proteins may induce apoptosis** of endothelial cells via expression of the apoptotic molecule Fas (CD95).

GENETICS/GENOMICS OF KIDNEY DISEASE IN THE SETTING OF HIV INFECTION

- APOL1 has been shown to be strongly associated with FSGS and HIVAN in African Americans.
- It is located on chromosome 22.
- APOL1 encodes apolipoprotein L1 which is involved in innate immunity.
- APOL1 kidney disease manifests as HIVAN or FSGS (NOS) with or without microcystic tubular dilatation.
- Genotyping the APOL1 G1 rs73885319 missense and G2 rs71785313 indel (i.e., insertion-deletion mutations) variants are sufficient to determine the risk of CKD.
- HIVAN is associated with low CD4 cell counts and often improves with effective ART.
- HIV-associated FSGS is associated with higher CD4 cell counts and occurs in patients undergoing ART.
- APOL1 high-risk genotypes are associated with progression to ESKD in HIV-positive patients.

Antiretroviral Therapy (ART) Nephrotoxicity

- HIV treatment guidelines recommend immediate initiation of ART in all HIV-positive individuals.
- Kidney function and CKD risk factors should be assessed prior to ART initiation.
- CKD risk scores have been developed to guide clinicians, although future studies are needed to determine their utility in diverse populations.
- Tenofovir disoproxil fumarate (TDF) is generally safe and well tolerated.
- However, there is an important potential for cumulative nephrotoxicity.
- Subclinical proximal tubular dysfunction (low-level proteinuria and excessive phosphaturia) is common.
- Approximately 1% to 2% of recipients develop treatment-limiting tubulopathy.
- Risk factors for tubulopathy include aging, immunodeficiency, diabetes, prolonged exposure, and concomitant use of didanosine or ritonavir-boosted protease inhibitors.
- Severe tubulopathy may progress to eGFR decline, osteomalacia, and pathological fractures.
- The newer pharmacoenhancer cobicistat also increases tenofovir exposure and may increase the risk of toxicity.
- Any drug (antiretroviral or other) may cause interstitial nephritis.
- New-onset eGFR decline or proteinuria should prompt a careful review of CKD risk factors and medications.
- Among antiretrovirals, atazanavir and indinavir have been most commonly linked to interstitial nephritis and nephrolithiasis.
- Studies have also linked atazanavir and lopinavir/ritonavir to rapid eGFR decline and incident CKD.
- Switching from ritonavir-boosted atazanavir or lopinavir to boosted darunavir has been associated with improved kidney function.
- The threshold for avoiding or discontinuing these agents may be influenced by local circumstances.
- In resource-limited settings, TDF dose adaptation may be an option.

RISK FACTORS FOR CKD

- Both HIV-related and traditional
- CKD risk factors influence CKD development and progression.
- With improved longevity among HIV-positive individuals, traditional CKD risk factors, particularly hypertension and diabetes, are of increasing concern worldwide.
- Hepatitis B virus (HBV) and hepatitis C virus (HCV) co-infections are associated with a 2- to 3-fold increased risk of progressive CKD.
- Other co-infections such as tuberculosis and syphilis may also contribute.

CKD SCREENING AND MONITORING

- CKD screening is recommended at the time of HIV diagnosis and ART initiation or modification.
- Urinalysis should be performed in all HIV-positive individuals to detect worsening or new onset of proteinuria or hematuria.

- Where feasible, quantification of proteinuria (spot urine albumin-to-creatinine or protein-to-creatinine ratio) should also be performed.
- More frequent monitoring is recommended in persons who are clinically unstable, severely immunocompromised, or viremic.
- Selection criteria for potential HIV-positive kidney transplant recipients in ESRD are shown in Table 45.1.

Table 45.1: Selection criteria for potential HIV-positive kidney transplant recipients

Meets standard criteria for kidney transplant recipients, plus the following:
Effective HIV suppression for >6 months before transplantation
⊥ Undetectable plasma HIV-1 RNA
⊥ CD4+ cell count >200 cells/mm^3
No active opportunistic infections
No history of:
⊥ Progressive multifocal leukoencephalopathy
⊥ Primary central nervous system lymphoma
⊥ Pulmonary aspergillosis
⊥ Visceral Kaposi's sarcoma
⊥ Coccidioidomycosis
⊥ Chronic intestinal cryptosporidiosis >1 months
Hepatology evaluation for patients co-infected with hepatitis B or hepatitis C virus

INVESTIGATIONS

- Nephrotic range proteinuria is usually present.
- Serum complement levels are normal.
- CD4 counts are variable, from normal to low.
- Presence of HIV antibodies is seen.
- Renal ultrasound usually shows echogenic kidneys with preserved or enlarged size of more than 12 cm in severe renal insufficiency.

TREATMENT

- The treatment is primarily comprising of the following:
  a. HAART
  b. ACE-inhibitors
  c. Steroids
- Benefit of HAART is believed to be due to the suppression of viral replication.
- Viral proteins/cytokines released during active viral replication directly cytopathic to kidneys.
- Recent evidence suggests 'non-viral' actions of HAART may be equally important.
- Protease inhibitors were shown to inhibit reactive O_2 species (ROS) generation and ROS-linked apoptosis of murine mesangial cells independent of HIV gene expression.

- This anti-apoptotic non-virologic effect of protease inhibitors may be important in humans.
- ACE inhibitors have been reported to show an increase in renal survival in cases of biopsy-proven HIVAN.
- Prednisone has been found in several studies to be associated with reduced risk of progressive renal failure with HIVAN.

PROGNOSIS

- The data regarding prognosis for patient survival after diagnosis of HIVAN are biased by the fact the majority of patients are referred to a nephrologist late in the course of their renal disease and HIV infection.
- Patients with HIVAN who are not treated with HAART, ACE-inhibitors, or prednisone, generally have a poor prognosis with a mean time to progression to ESRD of 1 to 3 months.
- Clinical variables associated with the progression of renal failure include:
  a. Elevated serum creatinine
  b. Low CD4 count
  c. High HIV viral load
  d. Higher level of proteinuria
  e. Previous antiretroviral therapy.

POINTS TO REMEMBER

- HIVAN which was first described 26 years ago, is an important cause of renal failure among HIV-positive patients.
- Since the introduction of HAART, the incidence of ESRD has decreased.
- HIV infects the renal epithelial cells.
- Renal epithelial cells are also a newly identified viral reservoir and a separate replicating compartment distinct from blood.
- Viral genes are necessary for causing renal disease.
- Treatment options, such as renal transplantation for HIV-infected patients are becoming realities.
- Despite improved survival with ART, HIV-positive individuals remain at increased risk for kidney disease.
- KDIGO has provided recommendations for the diagnosis, management, and prevention of kidney disease in this population, including a proposed histologic classification.

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Immunohistochemistry in Diagnosis of Thyroid Tumors

46

Swati Bhardwaj

INTRODUCTION

- The nationwide relative frequency of thyroid cancer is 0.1–0.2% (except Thiruvananthapuram; 1.99% among males and 5.71% among females) [Ref: ICMR, 2011)
- The age-adjusted incidence rates are 1 per 100,000 for males and 1.8 per 100,000 for females.
- Globally; Rapid increase in incidence from 1.3 per 100,000 in 1935 to 16.3 per 100,000 in 2008 has occurred.
- Need for performing IHC in thyroid lesions:
  - Lesions exhibiting equivocal features that make a distinction between benign and malignant difficult.
  - Interobserver or intraobserver disagreements in the diagnosis of follicular thyroid lesions.
  - Subjective and circumstantial application of diagnostic criteria.
  - Poorly differentiated or undifferentiated neoplasms.

CLASSIFYING IMMUNOHISTOCHEMICAL MARKERS IN THYROID

1. For thyroid-specific differentiation, i.e. to confirm that the tumor cells are of thyroid origin.
2. For differential diagnosis of various thyroid tumors, i.e. to resolve the confusion between differing entities in thyroid pathology.

IHC Markers for Thyroid-Specific Differentiation

TTF-1 (Thyroid Transcription Factor-1)

- Also named NKX2 homeobox 1(NKX2.1)
- 38 kDa nuclear protein
- It plays a crucial role in organogenesis and differentiation of thyroid and lung.
- Expressed in normal, benign, and malignant lesions.
- Helpful in determining thyroid or lung primary in the setting of metastases.
- TTF-1 positive non-pulmonary, non-thyroid carcinomas include: colorectal, ovarian, breast, endometrial, and endocervical adenocarcinomas (Table 46.1).

Table 46.1: Expression of TTF-1 in normal cells and various tumors	
Histopathological diagnosis	***Expression of TTF-1***
Normal thyroid follicular cells and parafollicular cells	100%
Papillary thyroid carcinoma	100%
Follicular thyroid carcinoma	100%
Follicular adenoma	100%
Medullary carcinoma	90%
Poorly differentiated carcinoma	90%
Undifferentiated carcinoma	None to 25%

- Two commercially available monoclonal antibodies: SPT24, and 8G7G3/1.
- The rate of aberrant TTF-1 expression in non-pulmonary non-thyroid carcinomas is higher for SPT24; greater than 10% in colorectal adenocarcinomas.
- With 8G7G3/1 clone, the aberrant expression rate is less than 2%.

Paired Box Gene 8 (PAX-8)

- PAX-8 is a member of the paired box family of transcription factors that is expressed during the organogenesis of the thyroid gland, Mullerian tract, and kidney.
- Mainly seen in thyroid and renal neoplasms, and infrequently in bladder tumors (Table 46.2).
- It is also seen in Mullerian origin malignancies, including ovarian malignant neoplasms.
- A few studies also report positive expression in thymic, parathyroid, and pancreatic neuroendocrine neoplasms.
- Normal tissues that express PAX-8:
  - Follicular cells of thyroid
  - Mullerian epithelial cells including endometrium, endocervix, and secretory cells of the fallopian tube
  - Renal tubular epithelium
  - Epithelial lining of the vas deferens
  - Islet cells of pancreas

Table 46.2: Expression of PAX-8 in various tumors	
Histopathological diagnosis	***Expression of PAX-8***
Papillary thyroid carcinoma	Nearly 100%
Follicular thyroid carcinoma	91 to 100%
Follicular adenoma	Nearly 100%
Poorly differentiated carcinoma	75 to 100%
Undifferentiated carcinoma	50 to 80%
Medullary carcinoma	Non-reactive with rare exceptions

Thyroid Transcription Factor-2 (TTF-2)

- Also named as forkhead box E1 (FOXE1)
- Plays a role in the development of negative controller of thyroid-specific gene expression (Table 46.3).

- TTF-2 expression is restricted to the thyroid, anterior pituitary, epithelia of oropharynx, trachea, and esophagus, exocrine cells of the seminiferous tubules of testis, epidermis, hair follicles.

Table 46.3: Expression of TTF-2 in various tumors

Histopathological diagnosis	*Expression of TTF-2*
PTC, FA, FTC	100%
Medullary carcinoma thyroid	75% in a focal staining pattern
Undifferentiated carcinoma	7%

Thyroglobulin (Table 46.4)

- It is a glycoprotein synthesized by thyrocytes, transported to the apical surface, secreted into the follicles, constituting the major component of colloid.

Table 46.4: Expression of thyroglobulin in various tumors

Histopathological diagnosis	*Expression of thyroglobulin*
Papillary thyroid carcinoma	100%
Follicular thyroid carcinoma	75 to 96%
Poorly differentiated carcinoma	57 to 92%
Undifferentiated and medullary carcinoma	Near lack of expression

IHC Markers Used in the Differential Diagnosis of Thyroid Neoplasms

Hector Battifora Mesothelial-1

- It is a membrane antigen found in the microvilli of mesothelial cells, normal tracheal epithelium, and adenocarcinoma of the lung, pancreas, and breast.
- Normal thyroid tissue shows virtually no expression.
- Benign lesions show lesser positivity and focal staining (Table 46.5).
- Malignant lesions show diffuse, strong expression. Overexpression of HBME-1 in a thyroid nodule is an indicator of malignancy!

Table 46.5: Expression of HBME-1 in benign lesions of thyroid

Thyroid lesions	*Expression of HBME-1*
Follicular adenoma	26%
Nodular goiter	12%
Lymphocytic thyroiditis	19%

Specificity and Sensitivity of HBME-1 in Malignant Lesions

- Sensitivity of HBME-1 in thyroid lesions:
  - 78.8% for thyroid malignancy
  - 87.3% for papillary thyroid cancer
  - 65.2% for follicular thyroid cancer
- Specificity of HBME-1 for malignancies = 82.1%

Cytokeratin 19

- It is a low molecular weight cytokeratin found in a variety of simple or glandular epithelia, both normal and neoplastic.
- However, CK-19 is normally not expressed in normal thyroid gland.
- CK-19 shows a higher expression in papillary thyroid carcinoma, although it is not specific.
- The specificity of CK-19 in papillary thyroid cancer is 63.1%.
- CK-19 is also expressed in lymphocytic thyroiditis, although the staining is only focal.
- If CK-19 4+ positivity is taken as a cut-off value to differentiate PTC from other mimicking lesions, it showed 77% sensitivity and 100% specificity and positive predictive value (Table 46.6).

Table 46.6 CK-19 expression sensitivity in thyroid lesions

Thyroid lesion	*CK-19 sensitivity*
Malignancy, in general	79.3%
Papillary thyroid cancer	82.2%
Follicular thyroid cancer	44.3%

Galectin-3

- Galectin-3 is a member of a family of beta-galactoside binding animal lectins shown to be involved in tumor progression and metastasis.
- It exists in two forms: phosphorylated and non-phosphorylated GAL-3.
- Phosphorylated form exists in both the cytoplasm and nucleus, and non-phosphorylated form resides exclusively in the nucleus.
- Galectin-3 plays an important role in cell-cell/cell-matrix adhesion, cell growth, neoplastic transformation/spread, cell cycle regulation/apoptosis, and cell repair processes.
- The specificity of GAL-3 in malignancy is 83.6% (Table 46.7).

Table 46.7: Expression of galectin-3 in thyroid malignancy

Thyroid lesion	*Sensitivity of GAL-3*
Malignancy, in general	84.6%
Papillary thyroid cancer	87.5%
Follicular thyroid cancer	72.6%

Trophoblastic Cell Surface Antigen 2

- It is also known as tumor-associated calcium signal transducer 2 (TAC-STD2)
- It is a transmembrane glycoprotein associated with tumor development and progression in a variety of epithelial carcinomas such as: Ovarian, colorectal, pancreatic, gastric, pulmonary, endometrioid endometrial, oral cavity squamous cell carcinoma.
- TROP2 overexpression is associated with tumor aggressiveness and poor prognosis (Table 46.8).

Table 46.8: TROP2 expression in thyroid lesions	
Thyroid lesion	***Expression of TROP2***
Normal thyroid tissue	Weak cytoplasmic staining, no membrane staining
Benign thyroid lesions	Rare, weak to moderate membrane staining
Follicular adenoma and follicular thyroid carcinoma	Focal weak staining in <5% of cases
Papillary thyroid cancer	Strong, diffuse membranous staining

- Thus, TROP2 is a potential novel biomarker for the identification of both classical and follicular variants of papillary thyroid cancer.
- It is even more specific than the three traditional markers! (CK19, HBME-1, Galectin-3).

Thyroperoxidase

- Thyroperoxidase is a thyroid-specific enzyme reflecting normal thyroid function.
- Lack of TPO expression is regarded as a marker of malignancy, with a specificity of 88%.
- The sensitivity of TPO for papillary thyroid cancer is 90%, and 76% for follicular thyroid cancer.

Calcitonin

- Calcitonin (also known as thyrocalcitonin) is a 32-amino acid linear polypeptide hormone that is produced primarily by the parafollicular cells (also known as C-cells) of the thyroid.
- Calcitonin expression is seen along with CEA, and chromogranin in medullary carcinoma thyroid.
- It is relatively specific for medullary carcinoma thyroid.

Fibronectins

- Fibronectins are extracellular matrix proteins produced by fibroblasts.
- They are involved in cell adhesion, migration, and tumor progression.
- Oncofetal FNs are isoforms of fibronectins that are highly expressed in fetal and neoplastic tissues, especially papillary thyroid cancer (Table 46.9).

Table 46.9: Fibronectin 1 expression in thyroid lesions	
Thyroid lesion	***Expression of fibronectin 1***
Normal thyroid tissue	0%
Benign thyroid lesions	5–6%
Papillary thyroid carcinoma	91%
Undifferentiated carcinoma	100%
Follicular thyroid carcinoma	50%
Hurthle cell carcinoma	75%

OTHER CELL ADHESION MOLECULES, CELL CYCLE REGULATORY PROTEINS, AND ONCOGENES

Cluster of Differentiation 44v6

- It is an isoform of CD44, a cell surface membrane glycoprotein that plays a role in the regulation of cell-cell, and cell-matrix interactions as well as cell migration.
- It is overexpressed in well-differentiated PTCs and FTCs, although expression is also seen in benign conditions at lower levels.

Cyclin Dependent Kinase Inhibitor 1B

- It is a nuclear cyclin-dependent kinase inhibitor.
- It plays a major role in controlling progression from the G1 to S phase.
- It is expressed strongly in normal thyrocytes, but loss of expression is seen in malignant conditions.

Cyclin D1

- Cyclin D1, a member of the family of cyclins, is a 36 kDa nuclear protein that functions as a regulatory subunit of cyclin-dependent kinases (CDK 4 and CDK 6), and controls progression of cell cycle from G1 to S phase.
- Overexpression of cyclin D1 is seen in 87.1% of malignant and 45.7% of benign thyroid lesions.

β-Catenin

- Normal thyroid follicular cells display strong membranous reactivity for beta-catenin.
- Nuclear expression of beta-catenin is seen in 32.1% of poorly differentiated carcinomas, 79.3% of undifferentiated carcinomas, and 0% of well-differentiated carcinomas.

IBRAF Mutation-Specific Antibody

- BRAF (the B-isoform of RAF kinase) oncogene is mutated in several types of tumors, such as colorectal adenocarcinoma, PTC, glioma, gastrointestinal adenocarcinoma, melanoma, and pulmonary adenocarcinoma.
- The most common mutation in BRAF is due to a T to A switch at position 1796, which results in an alteration from valine to glutamate at the V600E.
- The BRAF V600E point mutation has been reported in approximately 50% of PTCs.
- There is a higher frequency (approximately 70%) in tall cell variant and oncocytic variant of PTC and a much lower frequency (approximately 20%) in follicular variant of PTC.
- It is generally negative in benign follicular lesions, normal thyroid tissue, medullary carcinoma, and follicular carcinoma.
- PTC with the BRAF mutation is associated with a higher risk of recurrent, persistent disease, lymph node metastasis, and extrathyroidal extension.
- Many molecular techniques have been used to detect the BRAF V600E point mutation, including single-strand conformation polymorphism, mutation-specific polymerase chain reaction, direct gene sequencing.

- Two mutation-specific antibodies against BRAF V600E have become commercially available.
- First is VE1 clone (Spring Biosciences, Pleasanton, California).
- Other is anti-B-RAF mouse monoclonal antibody (New East Biosciences, Malvern, Pennsylvania).
- In general, BRAF mutation-specific antibody has been shown to be useful in detection of the BRAF V600 mutation, with a sensitivity and specificity of more than 95% when compared to other molecular methods.

Newer Techniques

Other newer techniques that may aid in tumor diagnosis in thyroid lesions are:
- Molecular markers
- Somatic mutations
- Gene expression
- microRNA analysis

MOLECULAR MARKERS

- Common molecular markers: GAL3, HBME1, BRAF, RET/PTC, PAX8, PPAR, hTERT, Telomerase, miRNA, multigene asssays
- Two multigene tests:
  - RNA-based 167 genes; 95% NPV
  - DNA-based.

Multigene Panels

Thyroseq V2.1

- 14 genes (point mutations)
- 42 gene fusions
- 8 gene expression
- Additional gene EIF1AX in case of DNA panel
- Sensitivity: 90.9%
- Specificity: 92.1%
- Overall accuracy: 91.8%

Suggested IHC Panels for Making Definitive Diagnosis

1. For establishing thyroid origin in case of undifferentiated neoplasms: TTF-1, Thyroglobulin, PAX-8.
2. For distinguishing benign from malignant lesions: HBME-1, GAL-3, TROP-2, CK19.
3. For predicting prognosis and aggressiveness: BRAF mutation-specific antibody, and to some extent TROP-2.

POINTS TO REMEMBER

- Morphological examination remains the cornerstone for the diagnosis of thyroid lesions.
- However, sometimes challenging cases with equivocal morphology are encountered.

- Ancillary studies, including somatic mutation testing, messenger RNA gene expression platforms, protein immunohistochemistry, and microRNA panels, are becoming increasingly important in these cases.
- Immunohistochemistry by far is the most commonly used technique out of these.
- However, there is no individual biomarker having sufficient sensitivity or specificity to distinguish benign from malignant lesions.
- However, HBME-1 is often strongly and diffusely expressed in PTC.
- It can be used as a marker (in a panel) to aid in the PTC diagnosis.
- Galectin-3 (in a panel) can be useful in differentiating malignant from benign thyroid lesions.
- CK19 has low sensitivity and specificity.
- Based on available information following IHC panels are recommended.
- For establishing thyroid origin in case of undifferentiated neoplasms: TTF-1, Thyroglobulin, PAX-8
- For distinguishing benign from malignant lesions: HBME-1, GAL-3, TROP-2, CK19
- For predicting prognosis and aggressiveness: BRAF mutation-specific antibody, and to some extent TROP-2
- The application of a panel of immunomarkers improves the differential power of individual markers and aids in the accurate classification of challenging thyroid derived lesions.

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Immunohistochemistry in Diagnosis of Liver Tumors

47

INTRODUCTION

- Liver tumor is the 5th most common cancer in men and 9th in women worldwide.
- It is the 2nd most common cause of death worldwide.
- The incidence of hepatocellular carcinoma in India is 3–5/100000.
- With improvements in imaging, specifically four-phase MDCT and dynamic contrast-enhanced MRI there has been a concomitant increase in the detection of small liver nodules.
- While many of these can be diagnosed on imaging, histology remains gold standard.
- It can be challenging to distinguish hepatocellular carcinoma (HCC) from other benign lesions particularly when presented with a small sample.
- Nonetheless, in recent years, there have been several advances reported employing immunohistochemistry to answer such questions.

ROLE OF IMMUNOHISTOCHEMISTRY

- IHC complements morphology in the characterization of liver tumors.
- It helps in differentiating between benign and malignant tumors in case of obscure morphology.
- Subtyping of hepatocellular adenoma (HCA).
- Differentiation between primary and metastatic tumors.

HEPATOCELLULAR ADENOMA (HCA)

- For discussion of hepatocellular adenoma, the reader is referred to Chapter 43.

FOCAL NODULAR HYPERPLASIA (FNH)

- Grossly, it presents as a well-circumscribed, unencapsulated, and nodular mass with a central fibrous zone.
- Microscopy shows nodules of hepatocytes, separated by fibrous tissue bands.
- Thick-walled arteries are seen.
- The intervening fibrovascular stroma often contains a lymphocytic infiltrate.
- Bile ductular structures showing bile ductular reaction and chronic inflammatory infiltrate are seen.
- On IHC glutamine synthetase (GS) shows map-like geographic positivity.

- The ductular reaction can be highlighted using CK7.
- The immunohistochemical distinction of FNH from HCA is discussed in Chapter 43.
- The differentiation between FNH, HCA and well-differentiated HCC is often subtle mainly due to inadequate biopsy.
- In this diagnostic problem IHC can play a crucial role.
- The first step is to perform glypican-3.
- If it is positive, then it goes in favor of well-differentiated HCC.
- Whereas, negative IHC favors FNH or HCA.
- However, it is to be kept in mind that if glypican-3 is negative, it does not always rule out hepatocellular carcinoma.
- This is because sensitivity of glypican-3 is very low in well-differentiated hepatocellular carcinoma.
- For this reason, more than one marker is always necessary.
- The sensitivity and specificity of glypican-3 for HCC has been reported to be 88% and 97% respectively.
- Another marker that can be useful is HSP-70.
- Diffuse positivity for HSP-70 favors HCC.
- On the other hand, negative HSP-70 points towards either FNH or HCA.

HEPATOCYTE DIFFERENTIATION MARKERS

HEP-PAR 1

- It marks cells of liver origin which could be either normal or neoplastic.
- In HCC it shows a granular cytoplasmic positivity.
- Sensitivity is 70% and specificity 84%.
- However, it should be kept in mind that positive staining can also be seen with gastric, lung, small intestinal, colonic, and pancreatic adenocarcinomas, cholangiocarcinoma, and melanoma (<15%).

Polyclonal CEA

- In both normal liver and HCC, it shows a canalicular positivity.
- Metastatic carcinoma can show any pattern except canalicular.
- It has high sensitivity and specificity for hepatocytes when a definite canalicular pattern is present.
- It is 70% sensitive and 100% specific for hepatocellular differentiation.

Arginase-1

- Novel marker of hepatocellular differentiation.
- It has been found to be more sensitive and specific than HepPar-1.
- It has a sensitivity of 84% and a specificity of 96% which is higher than HepPar-1.
- In HCC it shows cytoplasmic and nuclear positivity.
- ARG-1 expression has also been rarely reported in a small subset of pancreatic, colon and gastric cancers.
- But when HepPar-1 was also positive, the specificity rose to 100%.

APPROACH OF IMMUNOHISTOCHEMICAL MARKERS IN LIVER TUMOR

Diagnostic Panel of Immunostains for Hepatocellular Carcinoma

- It consists of the following three markers:
  1. Glypican-3
  2. HSP-70
  3. Glutamine synthetase
- If any two of these three markers are positive, then a diagnosis of HCC is given.
- This panel has sensitivity of 72% for resection specimen, 57% for biopsy and specificity of 100%.

IHC for Differentiating Premalignant from Malignant Lesions

- Following three markers can be employed:
  1. Glypican-3
  2. Glutamine synthetase (GS)
  3. HSP-70
- A positive glypican-3 goes in favor of HCC.
- The different patterns of glypican-3 expression in HCC seen are membranous, canalicular and cytoplasmic.
- Whereas a negative glypican-3 favors large regenerative nodule or a high-grade dysplastic nodule.
- Diffuse positivity for GS indicates HCC.
- On the other hand, patchy perivenular positivity indicates large regenerative nodule or a high-grade dysplastic nodule.
- Diffuse strong positivity for HSP-70 points towards HCC.
- While, absent/weak/patchy staining goes in favor of large regenerative nodule or a high-grade dysplastic nodule.

HCC VS. METASTATIC ADENOCARCINOMA

- The IHC panel useful for the distinction of HCC from metastatic adenocarcinoma is shown in Table 47.1.

Table 47.1: IHC panel for distinction of HCC from metastatic adenocarcinoma

IHC marker	*HCC*	*Metastatic adenocarcinoma*
Hep-par1	+	–
MOC-31	–	+
CK-7, 19, 20	–	+
GPC-3, p-CEA, Arginase-1	+	–

- Site specific markers such as TTF-1, CDX-2, ER, PR, GCDFP-15 and PSA are helpful to reach the primary site.

HCC VS. NEUROENDOCRINE TUMOR

- HCC can be difficult to distinguish from NET as both can form acinar and trabecular pattern and can be composed of tumor cells with abundant eosinophilic cytoplasm and round nuclei.

- Focal neuroendocrine differentiation is noted in HCC with the use of NSE, protein gene product 9.5, VIP, Calcitonin and S-100.
- But diffuse staining with Chromogranin and Synaptophysin strongly supports NET.

CLEAR CELL HCC VS. METASTATIC CLEAR CELL RCC

- The IHC panel useful for the distinction of clear cell HCC from metastatic clear cell RCC is shown in Table 47.2.

Table 47.2: IHC panel for differentiating clear cell HCC from metastatic clear cell RCC

IHC marker	*Clear cell HCC*	*Metastatic clear cell RCC*
Hep-par1, p-CEA, Arginase1	+	–
PAX-2, PAX-8, RCC marker, EMA, Vimentin, MOC-31	–	+

HCC VS ADRENOCORTICAL CARCINOMA

- The IHC panel useful for the distinction of HCC from metastatic adrenocortical carcinoma is shown in Table 47.3.

Table 47.3: IHC panel for differentiating HCC from metastatic adrenocortical carcinoma

IHC marker	*HCC*	*Metastatic adrenocortical carcinoma*
Hep-par1, p-CEA	+	–
Inhibin, Melan-A, Calretinin	–	+

FIBROLAMELLAR CARCINOMA

- Well-circumscribed, nodular and yellow/ brown with extensive fibrosis.
- A central fibrous scar, when present, resembles that of focal nodular hyperplasia.
- Microscopically, parallel fibrous lamellae separate cords and clusters of tumor cells with distinctive granular cytoplasm and large nuclei.
- IHC resembles classic HCC and expresses Hep-par1, p-CEA and Glypican-3.
- However, AFP is uniformly negative.
- CK-7 is expressed in nearly all of these tumors as compared to 10–30% of classic HCC.

HCC VS. CHOLANGIOCARCINOMA

- The IHC panel useful for the distinction of HCC from cholangiocarcinoma is shown in Table 47.4.

Table 47.4: IHC panel for distinction of HCC from cholangiocarcinoma

IHC marker	*HCC*	*Cholangiocarcinoma*
Hep-par1	+	–
MOC-31	–	+
CK-7,19, 20	–	+
GPC-3, p-CEA, Arginase-1	+	–

HCC VS. ANGIOMYOLIPOMA

- The IHC panel useful for the distinction of HCC from angiomyolipoma is shown in Table 47.5.

Table 47.5: IHC panel for distinction of HCC from angiomyolipoma

IHC marker	*HCC*	*Angiomyolipoma (epithelioid variant)*
Hep-par1	+	–
SMA, Desmin	–	+
HMB-45, Melan-A, Tyrosinase	–	+

Hepatoblastoma

- Grossly presents as a solitary mass with well-defined borders and variable nodularity, hemorrhage, necrosis, and bile staining.
- Fetal epithelial-type hepatoblastomas grow in cords of small hepatocytes that resemble adult liver cells
- "Light-and-dark" herringbone pattern due to admixed glycogenated (clear) and eosinophilic hepatocytes is seen.
- Embryonal-type hepatoblastoma shows cells having less cytoplasm than fetal type tumor cells, higher N: C ratios, poorly defined cell borders, and increased mitotic activity.
- Mixed-embryonal and fetal patterns can be seen.
- Embryonal areas appear less differentiated (immature) and fetal areas appear more mature.
- Mixed epithelial and mesenchymal components (such as osteoid or cartilage) are also described.

Differential Diagnosis

- Pure fetal hepatoblastoma can be histologically similar to HCA.
- However, alternating pink and white cytoplasmic staining pattern is typically not present in adenoma.
- HCA usually does not occur before 5 years of age except in association with metabolic disorder.
- GPC-3, Diffuse GS and Nuclear beta-catenin seen more often in HB than are in HCC.
- However, the marker which clinches the diagnosis is AFP as it is positive only in hepatoblastoma and negative in HCA.
- Hepatoblastoma with predominantly embryonal or small cell undifferentiated pattern can mimic the following tumors:
  - Wilms tumor
  - Neuroblastoma
  - Ewing sarcoma
  - PNET.
- **Diffuse GPC-3 expression** (embryonal HB) and **Nuclear beta-catenin** (embryonal and small cell undifferentiated) help in establishing the diagnosis of hepatoblastoma.

Calcifying Nested Stromal-Epithelial Tumor (CNSET)

- Morphologically, it shows epithelioid cells having hepatoid appearance along with a desmoplastic stroma.

- The IHC panel useful for the distinction of hepatoblastoma from CNSET is shown in Table 47.6.

Table 47.6: IHC panel for differentiating hepatoblastoma from CNSET

IHC marker	*CNSET*	*Hepatoblastoma*
Cytokeratin, Vimentin, CD56, SMA, CD117, NSE, WT-1	+	–
AFP	–	+

Infantile Hemangioma

- Microscopy shows irregular varying-sized vascular channels lined by endothelial cells.
- IHC for endothelial marker CD34 is confirmatory.

Epithelioid Hemangioendothelioma

- It shows a sinusoidal growth pattern with tumor cells present inside the dilated sinusoids.
- IHC for CD34 and CD31 helps in confirming the diagnosis.

Angiosarcoma

- Microscopic examination shows freely anastomosing vascular channels lined by atypical endothelial cells.
- On IHC these cells are positive for CD34.

RECENT ADVANCES

- Bile salt export pump (BSEP) is a recently described immunohistochemical marker for hepatocellular differentiation.
- It is a membrane-bound ATP binding cassette transporter expressed only in hepatocytes.
- It functions to transport bile out of the hepatocyte.
- A study has reported 90% sensitivity and 100% specificity for HCC.
- Furthermore, since it is expressed exclusively in hepatocytes, there is no requirement for a canalicular pattern of staining.
- MDR3 is another transporter protein against whom IHC is now available.
- A study has reported 83% sensitivity and 100% specificity for HCC.
- The combination of BSEP and MDR3 has been shown to be useful in differentiating hepatocellular carcinoma from cholangiocarcinoma and hepatoid carcinomas.
- An accurate tool enabling early diagnosis of HCC is clinically important since early detection of HCC markedly improves survival.
- In one study the authors have found 3 new immunohistochemical markers BANF-1, PLOD-3 and SF3B4 to HCC decision markers.
- They have superior capability to diagnose early-stage HCC in a large cohort of HCC patients as compared to the currently popular trio of HCC diagnostic markers Glypican-3, GS and HSP-70.

PROGNOSTIC MARKERS

- Expression of K13, EpCAM and CD13 in HCC is associated with aggressive biological behavior and poor clinical outcome compared to conventional HCC.

- CD13 is associated with early recurrence and poor prognosis and vimentin is associated with early recurrence.
- Expression of P53, VEGF and PCNA is associated with high recurrence and poor survival.
- The risk of recurrence or metastasis after surgery in an HCC patient with high GPC-3 expression was increased.

POINTS TO REMEMBER

- Diagnosing liver tumors can be challenging.
- It is especially so on needle biopsy specimens which may only minimally sample a lesion.
- Proper identification and classification is essential, as some lesions require no treatment at all.
- Whereas in others, resection, chemotherapy, and transplantation may be offered.
- Immunohistochemistry is complementary to morphology in the evaluation of liver tumors.
- It is necessary for subtyping of hepatocellular adenoma.
- Extensive sampling should be done to reach a final diagnosis and it is very difficult to interpret specially in needle biopsy where the sample is very less.
- In case of subtle morphology, immunohistochemistry plays an important role to differentiate between benign and malignant tumors.

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48 Immunohistochemistry Techniques: Basics and Recent Advances

Naveen

INTRODUCTION

- Immunohistochemistry is a combination of immunology and histology.
- It is defined as a technique for identifying cellular or tissue constituents (antigens) by means of antigen–antibody interactions.
- It also helps to determine the antigenic status of particular cells and microanatomic location of the antigen.
- IHC uses antibodies to distinguish antigenic difference between the cells.
- An antigen is a molecule that induces the formation of an antibody and bears one or more antibody binding sites.
- These are highly specific topographical regions composed of a small number of amino acids—***antigenic determinant groups or epitopes.***
- Antibodies are a class of serum proteins also known as immunoglobulins.
- The basic unit of each antibody is a monomer.
- An antibody can be monomeric, dimeric, trimeric, tetrameric, or pentameric.
- The monomer is composed of two heavy and two light chains, e.g. IgA, IgD, IgE, IgG, and IgM.

Antigen–Antibody Binding

- The amino acid side chains of the variable domain form a cavity which is geometrically and chemically complementary to a single type of antigen epitope.
- The analogy of a lock (antibody) and key (antigen) has been used.
- Antibody and antigen are held together by a combination of hydrogen bonds, electrostatic interactions and van der Waals forces.
- ***Affinity*** is the 3D fit of the antibody to its specific antigen.
- It is a measure of the binding strength between the antigenic epitope and its specific antibody-combining site.
- ***Avidity*** is functional combining strength of an antibody with its antigen.
- ***It is a*** property referring to the heterogeneity of the antiserum which will contain various antibodies reacting with different epitopes of the antigen molecule.

Principle of Immunohistochemistry

- IHC is a method for localizing specific antigen in tissues or cells based on antigen–antibody reaction.

- The site of antibody binding is identified either by tagging the antibody, directly or indirectly with a visible label like fluorescent dye, colloidal metal, hapten, radioactive marker, etc.

Types of Antibodies

- ***Polyclonal antibodies*** are a heterogeneous mixture of antibodies directed against various epitopes of same antigen.
- These are generated by different B cell clones of the animals which are immunochemically dissimilar.
- They are produced by immunizing an animal with a purified specific molecule (immunogen) bearing the antigen of interest.
- The animal will mount a humoral response and antibodies produced can be harvested from immunoglobulin-rich serum.
- The animal will produce numerous clones of activated plasma cells (polyclonal).
- ***Monoclonal antibodies*** are a homogenous population of Ig directed against a single epitope.
- They are generated by a single B cell clone from one animal which is immunochemically similar.
- They are produced by hybridoma technique which is a hybrid cell obtained by fusion of B lymphocyte with usually a tumor cell of myeloma.
- The hybrid cell has the capacity of antibody production derived from B cells as well as it can divide continuously because the property derived from myeloma cells.
- This technology ensures large-scale antibody production of single specificity.
- They are either cultured *in vitro* or passed through mouse peritoneal cavity to obtain monoclonal antibodies.

Detection Systems

- Antibody molecules cannot be seen with a light microscope or even with the electron microscope unless they are labeled or flagged by some method that permits their visualization.
- Detection systems attach labels or flags to primary or secondary antibodies to visualize the target antigen-antibody localization in the tissue sections.

Enzyme Labels

- Enzymes are the most widely used labels in IHC
- Horseradish peroxidase is the most widely used enzyme and in combination with the most favored chromogen, i.e. 3, 3α-diaminobenzidene tetrahydrochloride (DAB).
- HRP is commonly used as an antibody label for several reasons:
  - Its small size does not hinder the binding of antibodies to adjacent sites.
  - The enzyme is easily obtainable in a highly purified form.
  - Chance of contamination is minimized.
  - It is a stable enzyme and remains unchanged during manufacture, storage and application.
  - Endogenous activity is easily quenched.
- Endogenous peroxidase activity is present in a number of sites, particularly polymorphs and other myeloid cells.

- Any endogenous enzyme activity must be abolished to avoid false-positive reactions.
- Blocking by the hydrogen peroxide-methanol method is the most popular.
- Alkaline phosphatase (ALP) from calf intestine is the most widely used alternative enzyme.
- ALP is blocked by the addition of levamisole.
- Levamisole does not inhibit intestinal or placental ALP.
- 0% glacial acetic acid inhibits all types of alkaline phosphatase.
- Other labels used include bacterial-derived β-D-galactosidase, colloidal metal labels, Fluorescent labels and radiolabels.
- Chromogens DAB (3,3α-diaminobenzidene tetrahydrochloride) is the most widely used chromogen.
- It yields a crisp, insoluble, stable, dark brown reaction end-product.
- Although DAB has been reported to be a potential carcinogen, the risk is low.

IHC METHODS

Traditional Direct Technique

- The primary antibody is conjugated directly to the label.
- The conjugate may be either a fluorochrome or an enzyme.
- The technique is quick and easy to use.
- It provides little signal amplification and lacks sensitivity.

Two-step Indirect Technique

A labeling secondary antibody directed against the immunoglobulin of the animal species in which the primary antibody has been raised.

Advantages

1. Versatility is increased
2. The labeling process is applied only to the secondary antibody
3. The primary antibody can usually be used at a higher working dilution
4. The secondary antibody has a high order of specificity and affinity.

Polymer Chain Two-step Indirect Technique

- An unconjugated primary antibody, followed by a secondary antibody conjugated to an enzyme (HRP) labeled polymer (dextran) chain.
- This dextran chain has up to 70 enzyme molecules and 10 antibody molecules attached.

Advantages

1. Quick, reliable, and easily reproducible.
2. Great sensitivity.
3. Used for multi-color staining on single-slide preparations.
4. The most commonly used method in routine diagnostic use.

UNLABELED ANTIBODY-ENZYME COMPLEX TECHNIQUES (PAP AND APAAP)

Peroxidase Anti-Peroxidase (PAP)

- Complexes are formed from 3 peroxidase molecules and 2 anti-peroxidase.
- First layer of unconjugated primary antibody is used, e.g. rabbit anti-human IgG and the second layer of bridging antibody that is usually a swine anti-rabbit IgG.
- Peroxidase anti-peroxidase (PAP) complex used as 3rd layer in staining.

ALKALINE PHOSPHATASE ANTIALKALINE PHOSPHATASE METHOD / APAAP

- Same principle as PAP method except that it is replaced with an APAAP complex.
- Major applications in staining of tissues with high levels of endogenous peroxidase.
- Also used in double immunostaining in conjunction with peroxidase.

Immunogold Silver Staining Technique (IGSS)

- In this method the gold particles are enhanced by the addition of metallic silver layers to produce a metallic silver precipitate which overlays the colloidal gold marker.
- Silver lactate is the ion supplier and hydroquinone is the reducing agent.
- One major disadvantage is the formation of fine silver deposits in the background.

Avidin-Biotin Techniques

- A three-step technique, which has an unconjugated primary antibody as the first layer.
- It is followed by a biotinylated secondary antibody and the third layer is either a complex of enzyme-labeled biotin and streptavidin or enzyme-labeled streptavidin.
- The enzyme can be either HRP or ALP along with a chromogen of choice.

Major Disadvantages

1. Biotin has a high isoelectric point of approximately 10 and is therefore positively charged at neutral pH.
2. As a result, it may bind non-specifically to certain negatively charged structures such as cell nuclei.
3. Avidin, being a glycoprotein, also has a tendency to react with lectins via the carbohydrate moiety, again causing non-specific staining.

- These problems can be overcome by the substitution of streptavidin for avidin.
- Streptavidin has now largely replaced the use of avidin in IHC detection techniques.
- Streptavidin can be isolated from the bacterium Streptomyces avidin and like avidin it has four high affinity binding sites for biotin.

Advantages

- Biotin (vitamin H) is easily conjugated to antibodies and enzyme markers.
- Up to 150 biotin molecules can be attached to one antibody molecule, often with the aid of spacer arms.
- By spacing the biotins, the large streptavidin has room to bind and maximize its strong affinity for biotin.
- The streptavidin-biotin technique can employ either enzyme label bound directly to the streptavidin or the enzymes are biotinylated and the biotinylated label, forming the streptavidin-biotin complex.

Hapten Labeling Technique

- Bridging techniques using haptens such as dinitrophenol and arsanilic acid have been used.
- In this hapten is linked to the primary antibody and a complex is built up using an anti-hapten antibody and either hapten-labeled enzyme or hapten-labeled PAP complex.

Biotinylated Tyramide Signal Amplification

- First described by Bobrow et al in 1989.
- The technique is based on streptavidin biotin technique.
- Application of the primary antibody is followed by subsequent incubations in biotinylated secondary antibody and then either HRP labeled streptavidin or streptavidin-biotin-HRP complex.
- The critical stage is the subsequent treatment with the biotinylated tyramide amplification reagent.
- The bound peroxidase label in the presence of hydrogen peroxide catalyzes the biotinyl tyramide to form free biotin radicals.
- These reactive biotin molecules bind covalently to proteins adjacent to the site of the reaction.
- Further incubation in either horseradish peroxidase-labeled streptavidin or streptavidin biotin-horseradish peroxidase complex results in additional enzyme being deposited at the site of the reaction.

Advantages

- Enables many antigens which had previously been unreactive in formalin-fixed paraffin-embedded tissue to be demonstrated.
- Antibodies could be used at far greater dilutions.

Disadvantages

- Excessive background staining
- Biotin-free catalyzed signal amplification (CSA II)
- A biotin-free system to reduce the problems associated with endogenous biotin.
- Following incubation in primary antibody, the secondary anti-mouse immunoglobulin conjugated to horseradish peroxidase is added and the
- Third layer involves the deposition of fluorescein tyramide-amplification reagent.
- Finally reacted with peroxidase-conjugated anti-fluorescein, producing a greatly enhanced signal.

Unmasking of Antigen Sites

1. Proteolytic enzyme digestion
2. Microwave oven irradiation
3. Combined microwave oven irradiation and proteolytic enzyme digestion
4. Pressure cooker heating
5. Decloaker heating
6. Pressure cooker inside a microwave oven
7. Autoclave heating
8. Water bath heating
9. Steamer heating

Proteolytic Enzyme Digestion

- The principle of proteolytic enzyme digestion is breaking down formalin cross-linking and hence the antigenic sites for a number of antibodies are uncovered.
- The most common enzymes are trypsin and protease.
- Other enzymes include chymotrypsin, pronase, proteinase K, and pepsin.

Limitations

- Under-digestion—little staining, because the antigens are not fully exposed.
- Over-digestion—false-positive staining, high background levels, and tissue damage.

Heat-mediated Antigen Retrieval Techniques

- In formalin fixation intermolecular and methylene bridges and weak Schiff bases form intramolecular cross-linkages.
- These cross-linkages alter the protein conformation of the antigen, which may prevent it from being recognized by a specific antibody.
- Calcium complexes formed during formalin fixation prevent antibodies from combining with tissue-bound antigens.
- The hydroxymethyl groups and unreacted oxygen-rich groups (e.g. carboxyl or phosphoryl groups) can interact with calcium ions to produce large coordinate complexes which can mask epitopic sites by steric hindrance.
- The high temperature weakens or breaks some of the calcium coordinate bonds.
- The presence of a competing chelating agent at the particular temperature at which the coordinate bonds are disrupted removes the calcium complexes.
- Most commonly used chelating agent is citrate buffer and EDTA.

Pressure Cooker Antigen Retrieval

- Pressure cooking is more uniform than other heating methods.
- A pressure cooker at 15 psi (10.3 kPa) reaches a temperature of around 120°C at full pressure.
- It is this increased temperature that is a major advantage in unmasking nuclear tissue antigens such as Bcl-6, ER, PR, etc.
- It is preferable to use a stainless steel domestic pressure cooker, because aluminum pressure cookers are susceptible to corrosion from some of the antigen retrieval buffers.
- The pressure cooker should have a capacity of 4–5 liters, allowing a large batch of slides.

Microwave Antigen Retrieval

- Most domestic microwave ovens are suitable for antigen retrieval and operate at 2.45 GHz.
- A batch of 25 slides in a plastic staining rack can be irradiated at once. 25 slides in 600 mL of 0.01 M citrate buffer at pH 6.0.
- Irradiate on high power (800W) for 22 minutes
- Major disadvantage is uneven heating and hot spots.
- Other methods are infrequently used.

Pitfalls of Heat Pretreatment

1. Care should be taken not to allow the sections to dry after heating, as this destroys antigenicity.
2. The boiling of poorly fixed material also damages nuclear detail.
3. Fibrous and fatty tissues tend to detach from the slide.
4. This can be overcome by using Superfrost Plus microscope slides or Vectabond or APES-coated slides can be used.

Blocking Background Staining

- Non-specific uptake of antigen, particularly the high affinity of collagen and reticulin for immunoglobulins, can cause high levels of background staining.
- Tissues that give background staining as a result of hydrophobic interactions.
- This background staining can be blocked by:
  - Addition of the blocking serum to the diluted primary antibody
  - Addition of a blocking protein
  - Skim milk can be used
  - Addition of a detergent such as Triton X
  - Addition of a high salt concentration of 2.5% NaCl to the buffer.

RECENT ADVANCES

Enzyme-labeled Antigen Method

- It is widely used for research purposes only.
- This method was the ultimate in specificity among immunoperoxidase techniques.
- Only one antibody is used.
- The method exploits the fact that an antibody molecule possesses two valences, one of which may be bound to the antigen and the second valency left free to bind with additional molecules of antigen added subsequently.
- The additional antigen is presented in a form that is directly conjugated with HRP.

Advantages

1. Primary antibody can be impure
2. High-specificity
3. Double antibody techniques—to detect two antigens simultaneously within the same section by using two labeled antigens.

Disadvantage

The primary antibody is used at a relatively high concentration—not economical.

Double Labeling IHC Techniques

- Double labeling techniques permit the demonstration of two antigens concurrently within a single section.
- Lan and colleagues developed a simple, reliable, and sensitive method for multiple IHC.

- Cross-reaction of the second antibody with the first sequence may be eliminated by either using a double immunoenzymatic method in which two specific primary antibodies produced in two different species are used or
- Separate species-specific secondary antibodies coupled with different enzymes (e.g. peroxidase and alkaline phosphatase).

Multiplex Immunohistochemistry

- Multiplex IHC based on separate IHC protocols (four to eight or more) that are designed to detect different antigens in a tissue section.
- "Opal" (PerkinElmer) or "MultiOmyx" (NeoGenomics) the process taking two or more days.
- "UltraPlex" (Cell IDx) is the most recent technique that runs all reagents synchronously to complete a 4 or 10-plex in just 3 hours.
- The human eye cannot distinguish the resultant kaleidoscope of colors, so whole slide imaging and computer-based analysis is necessary.

Automation in IHC

The first automated devices for immunohistochemistry (IHC) appeared in mid-late 1980s. The reasons for the need in automation are:

- Less labor-intensive
- Reduced hands-on time
- Standardization
- High reproducibility
- Optimized use of reagents
- Improved error control.

Automated IHC Staining Principles

- Open individual slide system
- Liquid overlay technology
- Capillary gap staining
- Dynamic gap staining.

Open Individual Slide System

- The slides are arranged horizontally with reagents being applied using a dispenser.
- Closest to mimic manual staining.
- The principle has been used in Dako Autostainer Link 48 and intelliPATH from Biocare Medical.

Liquid Overlay Technology

- An inert fluid is deposited over the entire slide.
- Reagents are either overlaid or deposited into the overlay fluid.
- This technology allows reagent mixing on the slide.
- The overlay fluid restricts evaporation.
- Completed slides must be cleaned off the overlay fluid before coverslipping.
- This principle is currently being used by Ventana BenchMark systems.

Capillary Gap Staining

- The technology utilizes capillary forces to draw and/or keep liquid between two planar units that may be either two microscope slides with tissue facing each other, or a slide and a cover plate.
- The capillary gap system exists in a vertical version.
- Reagents are applied by placing the slides in a reagent jar and reagents are drawn out by blotting.
- Washing is performed by repeated reagent draw up and out.
- This principle is being used on Dako TechMate instruments (vertical orientation of slides).
- Also being used in Bond from Leica Biosystems (horizontal orientation of slide using an overlay convertible).

Dynamic Gap Staining

- The technology uses capillary forces to secure homogeneous spreading of reagent.
- Dynamic movement of the coverlid ensures continuous movement of reagents across the staining area.
- It provides homogeneous reaction conditions across the entire staining area throughout the full incubation time.
- Tight humidity control of the system facilitates use of elevated temperature which decreases incubation time.
- No slide cleaning is required post-staining.
- This principle is used in Dako Omnis staining instruments.

Types of Systems

1. Open systems
2. Closed systems

Open Systems

Pros

- Users may select any reagent
- Staining protocol is by preference
- Very high degree of flexibility
- Research application-friendly.

Cons

- Require highly skilled technicians
- Increased need for internal protocol optimization
- Reduced staining consistency.

Closed Systems

Pros

- High level of standardization through use of ready-to-use reagents
- High level of staining consistency

- Reduced hands-on time
- Flexibility in use of staff with varying skill sets.

Cons

- The visualization reagents and protocols are typically locked
- Reduced staining protocol options
- Antibody restrictions.

POINTS TO REMEMBER

- IHC testing is rapid, and relatively inexpensive compared with molecular methods.
- The introduction of prognostic and predictive markers in IHC has made a tremendously beneficial impact on patient diagnosis, treatment and prognosis.
- The detection of biomarkers by IHC in the era of "Targeted Therapy" has further solidified its important role in today's diagnostic pathology laboratory.
- Newer advancements like multiplex IHC will lead to development of novel applications of IHC.

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49 Intraductal Papillary Mucinous Neoplasm (IPMN) of the Pancreas

INTRODUCTION

- IPMNs are intraductal grossly visible (1 cm or more) epithelial neoplasm of mucin producing cells, arising in the main pancreatic duct or its branches.
- The neoplastic epithelium is usually papillary and shows variable mucin secretion, duct dilatation (cyst formation), and dysplasia.
- According to the WHO 2010 classification, they fall in the group of preneoplastic lesions.
- It was first described by Ohashi et al in 1982.
- They detected four patients with puffiness in the ampulla of Vater, dilated pancreatic ducts, and mucin secretion.
- They named it a "mucus-secreting pancreatic cancer".
- Sessa, an Italian pathologist coined the term intraductal papillary mucinous tumor (IPMT) in 1994.
- This was then renamed intraductal papillary mucinous neoplasm (IPMN) in 2000.

Epidemiology

- IPMN constitutes 5–7% of all pancreatic neoplasms.
- In India they constitute 3.2% of all pancreatic neoplasms.
- Amongst the exocrine neoplasms alone, IPMNs represented 4.8% of the cases in an Indian study by Kodiatte et al, while in the Western studies, it comprises 3% of all the cases.
- The pathognomonic feature for IPMN on pancreatoscopy is "Fish mouth ampulla".
- However, this feature is observed in only about 25–30% of cases.
- Peak age of incidence is 60–70 years.
- It is usually solitary, however, 20–40% of cases may be multifocal.
- Slightly higher prevalence in males has been observed.

CLASSIFICATION

- These tumors are classified on the following basis:
  - Location within gland.
  - Degree of cytological atypia.
  - Histomorphological features and immunohistochemical characteristics.

- According to their main location, IPMNs are classified into (Fig. 49.1):
  - Main duct (MD) type (16–36%)
  - Branch duct (BD) type (40–65%)
  - Mixed or combined type (15–23%).
- This classification has important prognostic implications.
- Most main duct type and combined type IPMNs exhibit malignancy (i.e., high-grade dysplasia or carcinoma), with about 45% having an associated invasive carcinoma.
- In contrast, most branch duct types show low-grade dysplasia and only about 15% are associated with invasive carcinoma.
- The natural history of branch duct type under 30 mm in size and without mural nodules is particularly favorable.

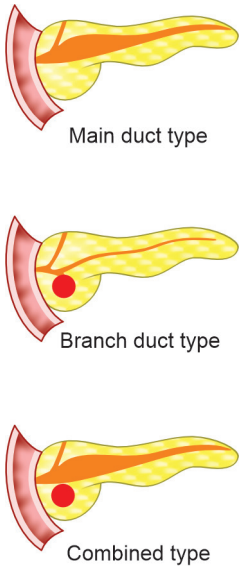

Fig. 49.1: Classification of IPMN based on location

Main Duct Type IPMNs

- At external examination, the pancreas may be thickened in the affected area.
- Cut surface shows dilatation of the main duct with irregular outline and lumen filled by mucus and villous or papillary projections.
- The rest of the pancreas shows appearance of obstructive chronic pancreatitis due to pancreatic duct obstruction.
- They are mostly located in the pancreatic head but 1/3rd occur in the body and tail and 5% in the entire main pancreatic duct (diffuse main duct type).
- The affected duct is markedly dilated, and there is often a polypoid nodule filling the lumen of the proximal segment of the duct.
- Branch duct IPMNs are usually cystic with a smooth and glistening cyst lining.
- According to the degree of cytological atypia IPMN is classified into the following three categories:
  - IPMN with low-grade dysplasia—neoplastic cells form a single layer of well-oriented small, uniform nuclei. They lack frequent mitoses.
  - IPMN with intermediate-grade dysplasia shows features intermediate between low-grade and high-grade dysplasia.
  - IPMN with high-grade dysplasia—it shows complex architecture with a cribriform pattern, nuclear pleomorphism and increased mitotic figures.
- Based on the histomorphological features and immunohistochemical characteristics IPMNs are classified into:
  - Gastric
  - Intestinal
  - Pancreatobiliary
  - Oncocytic.
- The oncocytic type was originally described as a separate entity (intraductal oncocytic papillary neoplasm).
- The current (2010) WHO puts this neoplasm under the general category of IPMNs.

Gastric-type IPMNs

- The papillae are lined by a simple epithelium that resembles gastric foveolar epithelium.
- Branch duct IPMNs often show gastric foveolar differentiation.
- Gastric intraductal papillary mucinous neoplasm has a multicystic appearance.
- The lining epithelium shows columnar cells with a basal nucleus and apical mucin.
- Scattered goblet cells may be seen.
- Immunohistochemical staining is positive for MUC5AC.
- MUC2 highlights the goblet cells.

Intestinal-type IPMNs

- It shows papillae lined by pseudostratified columnar cells with cigar-shaped nuclei and apical goblet-like mucin.
- Most main duct IPMNs are of intestinal type.
- Immunohistochemically they show MUC2 expression.

Pancreatobiliary-type IPMNs

- They usually involve the main pancreatic duct.
- Characteristically show more complex, interconnecting papillae lined by atypical cuboidal cells that have little intracellular mucin.
- They have enlarged round nuclei and a prominent eccentric nucleolus.
- Immunohistochemical expression of MUC1 is seen.

Oncocytic-type IPMNs

- They exhibit unilocular or multilocular papillary ducts composed of arborizing papillary structures with focal cribriform pattern.
- Papillae are lined by stratified and pseudostratified oncocytic cuboidal cells with intraepithelial lumina.
- Often, the papillary cores show edematous changes.
- Oncocytic cells have abundant eosinophilic cytoplasm, round nuclei with fine chromatin, single prominent eccentric nucleolus and intraepithelial lumina formation.
- The immunohistochemical differences between various subtypes of IPMN are shown in Table 49.1.

Table 49.1: Immunohistochemical differences between various subtypes of IPMN

	Pan CK	*MUC1*	*MUC2*	*CDX2*	*CK20*	*MUC5AC*	*MUC6*
IPMN							
Gastric type	+	–	–	–	–	+	–
Intestinal type	+	–	+	+	+	+	–
Pancreatobiliary type	+	+	–	–	–	+	+
Oncocytic type	+	+	–	–	–	+	+

Invasive Carcinoma Arising from IPMN

- 15 to 40% of cases of IPMN are associated with invasion at presentation.
- Two main histologic types are seen, namely:
  - Colloid carcinoma
  - Tubular adenocarcinoma
- Other types, such as an oncocytic type are also seen.
- Pathologic analysis indicates that intestinal-type IPMN can progress directly to an unusual subtype of invasive pancreatic cancer known as colloid carcinoma.
- In addition, a subset of, typically gastric- (and pancreaticobiliary) type, progress to invasive tubular carcinomas, which are histologically indistinguishable from classic pancreatic ductal adenocarcinoma (PDA).
- Recent studies have shown that IPMN-derived PDA accounts for ~10% of all PDA resected.
- Thus ~90% of PDA are considered to originate from pancreatic intraepithelial neoplasia (PanIN).
- The precise origins of concomitant PDA however, remains to be fully determined.
- Genetic alterations during progression of IPMN and PanIN to invasive carcinoma
- It is clear from above that pancreatic ductal adenocarcinoma (PDA) can arise from either progression of IPMN or PanIN.
- It is not known whether early IPMNs (incipient IPMNs) originate from low-grade PanIN develop independently from normal pancreatic ducts or other pancreatic cell lineages.
- The current understanding of the molecular pathway is presented in Fig. 49.2.
- The precise timing of RNF43 mutations and the order of GNAS and KRAS mutations have not been fully established.

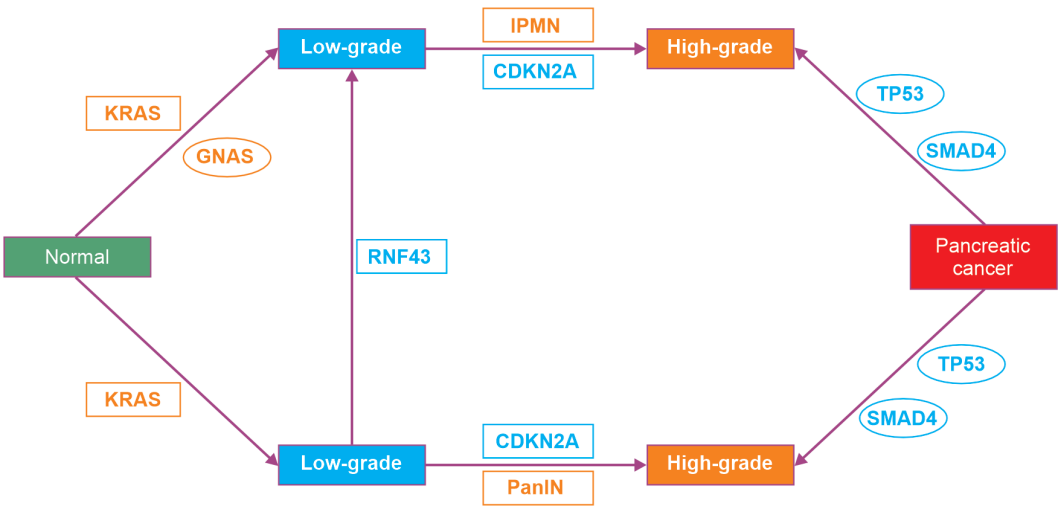

Fig. 49.2: Molecular pathway for progression of IPMN and panIn to pancreatic ductal adenocarcinoma. Orange boxes depict oncogenes and blue boxes tumor suppressor genes.

- The frequency of mutations in the various genes are shown in Table 49.2:

Table 49.2: Frequency of various genetic mutations in IPMN

Genetic alteration	*Frequency*
KRAS mutation	38–100%
Loss of p16	78%
P53 mutation	50%
SMAD4/DPC4 expression	In almost all of noninvasive IPMNs
Loss of SMAD4/DPC4 expression	10% of colloid carcinoma
GNAS mutation	66%
STK11/LKB1 gene inactivation	25%

Colloid Carcinoma

- The characteristic morphologic feature is invasive neoplastic cells floating in pools of mucin.
- Associated intraductal papillary mucinous neoplasm may be seen.
- Immunohistochemically MUC2 expression and CDX2 nuclear expression is seen.

Distinction Between IPMN and PanIN

- PanIN is not clinically or radiographically evident, and there is no macroscopically identifiable lesion.
- It is often recognized as a microscopic abnormality.
- There is a significant overlap in terms of morphology and molecular abnormalities between small IPMN and PanIN.

Fukuoka Guidelines

- Lesions less than 0.5 cm in diameter are categorized as PanIN.
- It should be noted that when this 0.5 cm in diameter rule is used, no adjacent IPMN lesion should be present, as the latter can involve smaller branches of the duct, mimicking a PanIN lesion.

Diagnostic Evaluation of IPMN

Clinical Presentation

- IPMNs are usually asymptomatic and often discovered incidentally on imaging.
- When symptomatic they present with the following features:
  - Epigastric discomfort or pain (70–80%)
  - Nausea and vomiting (11–21%)
  - Backache (10%), weight loss (20–40%), diabetes, and jaundice.

Other Investigations

- USG abdomen has only limited value.
- CT scan/MRI are the gold standard.
- Endoscopic ultrasound shows the following findings:
  - Dilation of MPD
  - Hypoechoic thickening of duct wall

- Mural nodules or papillary projections
- Pancreatic atrophy
- Multiple cysts communicating with main duct (not dilated)—branch duct IPMN

- ERCP is often supplanted by MRCP.
- MRCP:
  - Non-invasive, diagnostic method with fewer procedure-related risks (compared to ERCP).
  - More specific than ERCP in imaging pancreatic duct anatomy.
  - Bunch of grapes appearance—branch duct IPMN.
- **Fine-needle aspiration cytology (FNAC)**—FNAC can be performed in the following ways:
  - Endoscopically—most commonly endoscopic ultrasound (EUS) guided.
  - Percutaneously—CT-guided/ US-guided
- Analysis of aspirates includes the following:
  - Cytology
  - Viscosity
  - Enzymes (amylase and lipase)
  - Antigenic tumor markers.

Cytology

- IPMNs show papillary clusters lined by mucin-containing columnar cells.
- Mucinous cystic neoplasms (MCNs), on the other hand, show mucin-containing columnar cells with rarely papillary sheets.
- Presence of malignant cells on FNA is highly specific for mucinous cystadenocarcinoma or IPMN carcinoma.
- However, the diagnostic accuracy of cytology is quite poor (59%).
- The main limitation is low cellularity of the aspirate.
- EUS-guided Tru-cut biopsy has been evaluated in a small sample size and has been found to be safe.
- The findings of an analysis of aspirate in common cystic lesions of pancreas are summarized in Table 49.3.

Table 49.3: Analysis of aspirate in common pancreatic cystic lesions

	SCNs	*MCNs*	*IPMNs*
Cytology	Cuboidal, glycogen rich cells	Columnar cells	Columnar cells, papillary clusters
Fluid appearance	Thin, clear	Viscous, clear	Viscous, clear
Mucin	Absent	Present	Present
Amylase	Low	Low	High
CEA (ng/mL)	< 5	> 192	> 192
Malignant potential	No	Yes	Yes

Clinical Challenge

- The main challenge in these cases is to establish which IPMNs can be managed by clinical and radiological follow-up without requiring surgical excision and which should be removed because they are likely to develop invasive cancer.

- To help make clinical decisions Sendai criteria and Fukuoka guidelines were formulated.
- First published in 2006 (Sendai criteria) and updated in 2012 (the Fukuoka guidelines) were arrived at on the basis of a consensus symposium held during the 14th meeting of the International Association of Pancreatology in Fukuoka, Japan, in 2010.
- In these guidelines certain worrisome and high-risk features were described which are shown in Table 49.4.

Table 49.4: High risk and worrisome features in the clinical evaluation of IPMNs

Worrisome features (moderate-risk)	*High risk stigmata (high-risk)*
Clinical evidence of pancreatitis	Obstructed common bile duct associated with lesion of the pancreatic head
Cyst size >3 cm	Enhanced solid component within the cyst
Thickened/enhanced cyst walls	Main pancreatic duct size ≥10 mm
Main pancreatic duct size 5–9 mm Non-enhanced mural nodules Abrupt change in main pancreatic duct caliber, with distal pancreatic atrophy Regional lymphadenopathy	

The major changes in these guidelines are as follows:

1. *Classification*

- **1a:** The threshold of MPD dilation, segmental or diffuse, for characterization of MD-IPMN has been lowered to >5 mm without other causes of obstruction.

2. *Investigation*

- **2a:** CT or MRI with MRCP is recommended for a cyst of "1 cm to check for "high-risk stigmata", including enhanced solid components and MPD size of "10 mm, or "worrisome features".
- **2b:** MDCT and MRCP are most useful for distinguishing BD-IPMN from other cysts by showing multiplicity and a connection to the MPD.

3. *Indications for resection*

- **3a:** Resection is recommended in all surgically fit patients with MD-IPMN.
- **3b:** A BD-IPMN of >3 cm without "high-risk stigmata" can be observed without immediate resection.

4. *Methods of resection and other treatments*

- **4a:** Pancreatectomy with lymph node dissection remains the standard treatment for invasive and non-invasive MCNs and IPMNs.
- **4b:** Multifocal BD-IPMNs carry a similar risk of malignancy to unifocal BD-IPMN.

5. *Histological aspects*

- **5a:** The type of invasive carcinoma that is whether colloid or tubular has major prognostic implications and should be part of the reporting of IPMNs, with colloid carcinomas being characterized by "intestinal" differentiation and a better prognosis than tubular carcinomas.
- **5b:** Instead of "minimally invasive carcinoma" derived from IPMN or MCN, it would be more appropriate to stage invasive carcinomas with the conventional staging protocols and further substage the T1 category into:
  - T1a (≤0.5 cm),
  - T1b (>0.5 cm and ≤ 1 cm), and
  - T1c (1–2 cm).
- **5d:** If clear high-grade dysplasia or invasive carcinoma is present at the margin by frozen section analysis, further resection is warranted.

  All patients should be informed preoperatively about the possibility of total pancreatectomy. Moderate-grade or low-grade dysplasia may not require any further therapy.
- **5e:** Pathologists should make every attempt to classify the lesion as MD-IPMN or BD-IPMN, being careful to identify the MPD as precisely as possible when processing the specimen.

6. *Methods of follow-up*

- **6a:** Patients without "high-risk stigmata" should undergo MRI/MRCP (or CT) after a short interval (3–6 months) to establish stability, and then annual history/physical examination, MRI/MRCP (or CT) and serologic marker surveillance.

  Short interval surveillance (3–9 months) should be considered for patients whose IPMN progresses toward "high-risk stigmata" and patients with a family history of hereditary PDAC.
- **6b:** Non-invasive MCNs require no surveillance after resection. IPMNs need surveillance based on the resection margin status. If there are no residual lesions, repeat examinations at 2 and 5 years may be reasonable.

  For patients with low-grade or moderate-grade dysplasia at the margin, surveillance at least twice a year is suggested. The follow-up strategy of invasive IPMN should be identical to that for PDAC.

POINTS TO REMEMBER

- Understanding of IPMNs is continuously evolving.
- IPMNs constitute less than 10% of all pancreatic neoplasms, with MD-IPMN being the commonest.
- If untreated, some of them may follow the dysplasia-carcinoma sequence, progressing into invasive carcinoma.
- While the recent guidelines help in managing patients with IPMN, various parameters stratifying the risk for malignancy are being constantly analyzed.
- More extensive research is needed in the future for a better understanding of this relatively new pathological entity.

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Lesions of the Intermediate Trophoblast

50

INTRODUCTION

- Gestational trophoblastic disease (GTD) means different things to the pathologist and the clinician.
- The pathologist utilizes a classification based on histopathology, while the clinician uses a clinically based classification.
- There is no need every time to submit tissue for histopathology to appropriately manage the patient.
- Amongst these diseases lesions of the intermediate trophoblast represent a diagnostically challenging group.

TYPES OF TROPHOBLASTS

There are primarily three types of trophoblasts.

- **Previllous**
  - These are responsible for the initial invasion of maternal tissues during the early stages of implantation and for further invasion and expansion of the implantation site.
- **Villous**
  - These comprise the cytotrophoblast (CT), syncytiotrophoblast (ST), and intermediate trophoblast (IT).
  - Amongst these intermediate trophoblasts are very scant.
- **Extravillous**
  - They are used to make non-villous parts of the placenta, e.g. chorion laeve.
  - They infiltrate the decidua, myometrium, and spiral arteries of the placental site.
  - They are composed of exclusively IT.

MORPHOLOGY OF TROPHOBLASTIC CELLS

Cytotrophoblasts

- They are small primitive epithelial cells that are uniform, and polygonal to oval in shape
- Cytotrophoblastic cells have a single nucleus, clear to granular cytoplasm, and well-defined cell borders.

- Mitotic activity is evident.
- They form the germinative component of villous cells.

Syncytiotrophoblasts

- These cells form a large, multinucleate cellular mass with dense amphophilic cytoplasm often containing multiple vacuoles that vary in size.
- A distinct brush border often lines the cell membrane.
- Nuclei are dark and often appear pyknotic.
- They do not show mitotic activity.
- They form the differentiated component of villous cells.

Intermediate Trophoblasts

There are three types of intermediate trophoblasts.

- **Villous type IT:**
  - They are seen in the form of trophoblastic columns.
  - On IHC they are HNK-1 positive.
  - HNK-1 is also known as Leu-7 and CD57.
- **Implantation site IT:**
  - These cells invade and replace the spiral arteries of the implantation site (basal plate) to establish the maternal/fetal circulation
  - IHC—Mel CAM and hPL positive.
  - They show lower proliferative activity and are limited to early pregnancy.
  - These cells invade only the inner third of the myometrium in the first trimester, decreasing to less than 10% of the myometrium by term.
  - Fusion of these cells into multinucleated cells leads to the loss of their invasive phenotype.
  - Eosinophilic fibrinoid material is often deposited around implantation site intermediate trophoblast.
- **Chorionic type IT:**
  - They are seen away from chorionic frondosum.
  - On IHC these cells are PLAP positive.
  - Proliferation occurs throughout gestation.
  - They are responsible for the synthesis of extracellular matrix which is required to maintain the tensile strength of the fetal membrane.
  - Chorionic type IT form the biological and mechanical barrier to the maternal immune system and are important for fetal allograft survival.

The above features are summarized in Table 50.1.

Table 50.1: Summary of morphological and immunohistochemical features of various types of trophoblastic cells

Morphology	*Cytotrophoblast*	*Intermediate trophoblast*			*Syncytiotrophoblast*
		Villous	*Implantation site*	*Chorionic type*	
Nucleus	Single	Single	One to several	Single	Multiple
Shape	Round	Polyhedral	Variable	Round to polyhedral	Irregular

Contd.

Table 50.1: Summary of morphological and immunohistochemical features of various types of trophoblastic cells

Morphology	*Cytotrophoblast*	*Intermediate trophoblast*			*Syncytiotrophoblast*
		Villous	*Implantation site*	*Chorionic type*	
Cytoplasm	Scant, clear to granular	Abundant, eosinophilic to clear	Abundant, amphophilic	Abundant, eosino-philic	Abundant, dense, multiple vacuoles
IHC					
Cytokeratin	++++	++++	++++	++++	++++
hCG	–	–	+ (multi-nucleated)	–	++/++++
hPL	–	–/+	++++	++	++/++++
Mel-CAM	–	–/++++	++++	++	–
p63	++++	–	–	+++	–
HNK-1	–	++++	–	–	–
EMA	–	–	–	+++	–
Ki-67	30%	>90%	0	3–6%	0

Normal Placenta

- Size at term is 450 to 630 grams
- Diameter is 15 to 25 cm
- It is up to 3 cm thick
- Ovoid to round in shape, single lobe with 15 to 20 cotyledons
- Membranes are clear and inserted at the margins
- Placental parenchyma is beefy red without any lesions
- Chorionic plate is clear with uniform size vessels
- Umbilical cord is usually inserted eccentrically with the presence of three blood vessels
- It is 1–2 cm in thickness. Uniform white in thickness with the presence of Wharton's jelly.

GESTATIONAL TROPHOBLASTIC DISEASES

Epidemiology

- Reported incidence is 1:160 in India
- They are more common in blacks and oriental people
- Reproductive age group is affected
- More common in persons from low socioeconomic background.

Placental Site Trophoblastic Tumor (PSTT)

- It is a neoplasm of implantation site intermediate trophoblastic cells (IT)
- Originally it was termed atypical chorioepithelioma by Marchand in 1895
- They comprise <3% of all GTD cases
- Beta hCG levels are lower than choriocarcinoma.

Pathogenesis

- Normal implantation site shows a regulated invasion by IT.
- In PSTT there is a dysregulated invasion leading to deep penetration into myometrium.
- It resembles trophoblastic infiltration of endomyometrium in early pregnancy.
- Genetically the tumor cells are **Diploid** and do not demonstrate significant karyotypic abnormalities.
- >85% of cases are associated with an antecedent female gestation and show lack of Y chromosome.
- This points toward the role of paternal X chromosome and derivation from the previous molar gestation.
- These cells show expression of activated MAP kinase.
- RAS/RAF/MEK pathway is also involved in the following steps:
  - Proliferation
  - Differentiation
  - Apoptosis
  - Angiogenesis and migration.

Clinical Presentation

- Age group ranges from 19 to 62 years of age with an average of 30 years.
- Parous women with a previous history of term pregnancies, abortions and hydatidiform mole are at an increased risk.
- The patients present with amenorrhea, abnormal uterine bleeding with uterine enlargement.
- When uterine enlargement ceases it leads to a missed abortion.
- Rarely the patients may have virilization and nephrotic syndrome.
- Since the radiological features are non-specific definite diagnosis relies on histopathology.
- Samples that are often submitted include:
  - Endometrial curettage
  - Hysterectomy specimen
  - Biopsies from other sites.

Gross

- It is highly variable.
- These tumors are usually well-circumscribed but may also be poorly demarcated.
- They may be polypoid or infiltrative.
- Cut surface is soft and tan with or without areas of hemorrhage and necrosis.
- Extension into serosa is a common finding leading to perforation with or without extension to broad ligament.

Microscopy

- The tumor cells are arranged in the form of confluent sheets (Fig. 50.1).
- However, at the periphery of the tumor the intermediate trophoblastic cells invade singly or in cords and nests.
- They are seen separating individual muscle fibers and groups of fibers.
- The tumor cells are large, polygonal implantation site intermediate trophoblastic cells.

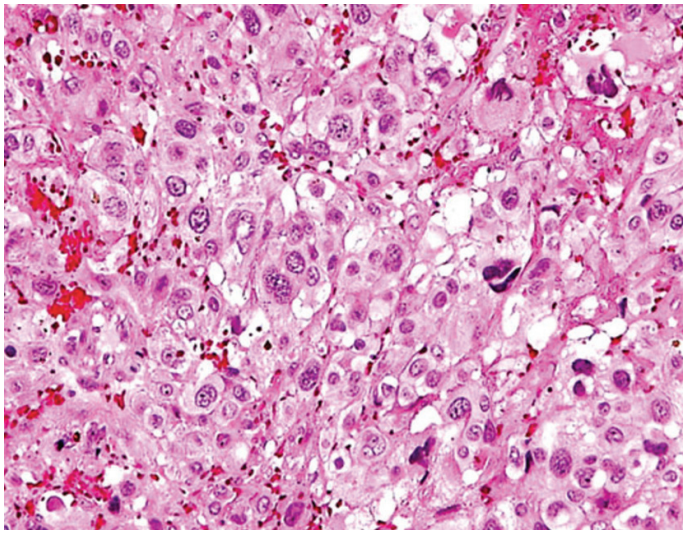

Fig. 50.1: Sheets of large, polygonal implantation site intermediate trophoblastic cells with irregular, hyperchromatic nuclei and dense eosinophilic to amphophilic cytoplasm with occasional vacuoles

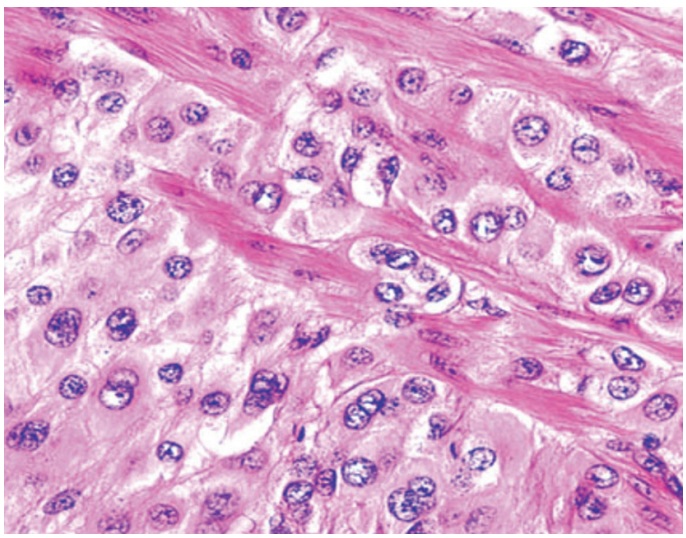

Fig. 50.2: Tumor cells infiltrating myometrial smooth muscle

- They have a dense eosinophilic to amphophilic cytoplasm with occasional vacuoles Irregular, hyperchromatic nuclei.
- The tumor cells may assume a spindle shape near the myometrium and may also show multinucleated IT cells.
- Decidual or an Arias-Stella reaction may be present in the adjacent, uninvolved endometrium.
- **However, villi are not identified.**
- The tumor cells invade the vessel wall and replace it entirely.
- Fibrinoid material is seen in and around the vessels.

IHC

- The tumor cells are immunoreactive to the protein markers expressed in implantation site intermediate trophoblast:
  - hPL
  - CD146 (Mel-CAM)
  - HLA-G
  - CD10
  - Mucin-4.

Aggressive PSTT

- It shows sheets of tumor cells with clear or amphophilic cytoplasm.
- They have atypical nuclei and extensive necrosis.
- Mitotic count is >5/10 hpf.

Differential Diagnosis

- Exaggerated placental site
- Choriocarcinoma
- Epithelioid trophoblastic tumor
- Epithelioid smooth muscle tumor
- Others: Carcinoma, carcinosarcoma, melanoma
- Exaggerated placental site:
  - It is the most difficult differential since both lesions are characterized by an exuberant infiltration of implantation site intermediate trophoblastic cells.
  - Immunophenotype of both lesions is similar.
  - Exaggerated placental site, is however, **microscopic in size, lacks mitotic activity,** composed of intermediate trophoblastic cells separated by masses of hyaline, and usually shows **admixed decidua and chorionic villi.**
  - It shows a larger number of multinucleated trophoblastic cells than PSTT.
  - Serum hCG level returns to normal following curettage.
- Choriocarcinoma
  - High elevation of serum hCG: Tens of thousands (mIU/mL).
  - Biphasic trophoblastic population with marked hemorrhage and necrosis.
  - Absence of fibrinoid material in and around vessels.
- Epithelioid leiomyosarcoma
  - Normal serum hCG level.
  - Lacks fibrinoid material in and around vessels.
  - Cytokeratin, hCG and HPL are negative.
  - SMA, desmin and caldesmon are positive.
- Poorly differentiated carcinoma, carcinosarcoma, melanoma
  - The infiltrative pattern of PSTT within the myometrium is not seen.
  - Vascular invasion and the deposition of fibrinoid material are not seen.
  - IHC - hCG and HPL are negative.
  - Instead, respective markers for these tumors are positive.

Clinical Behavior

- Most cases are self-limited
- Perforation at the time of curettage may occur.

- Metastatic deposits are seen in lung, liver, abdominal cavity, and brain.
- Monitoring is done by serial measurements of Beta-hCG or Beta core fragment of Beta-hCG

Prognosis

- It depends on the FIGO stage.
- Adverse prognostic factors include:
  - Metastatic involvement
  - Long interval from the antecedent pregnancy (48 months)
  - Age >35
  - hCG >1,000 mIU/mL
  - Depth of invasion
  - p53 immunoreactivity
  - Ki67> 50%.

EPITHELIOID TROPHOBLASTIC TUMOR

- Rare type of GTN, that resembles poorly differentiated carcinoma
- Neoplastic transformation of chorionic type IT
- Originally termed 'atypical carcinoma'
- Beta HCG: always elevated
- Primary tumor most commonly occurs in the *endomyometrium*
- Other sites:
  - Uterine cervix
  - Broad ligament, FT
  - May even present as a primary lung tumor (simulating squamous and pleomorphic carcinomas).

Pathogenesis

- Not known.
- Hypothesis: Some placental site nodules may represent a stage in tumor progression to ETTs.
- Cyclin E may have a role.

Clinical Features

- Reproductive age group (Mean: 38 years, may be seen after menopause)
- Presents with abnormal vaginal bleeding
- May present with metastasis
- Diagnosis is based on clinical history and Beta HCG levels.
- Radiology: For ruling out metastasis.
- USG: Well-circumscribed mass with sharp borders and peripheral halo of low echogenicity.

Gross

- Solitary, discrete nodules that deeply invade the cervix or myometrium
- Cut surface: Solid with cystic areas, tan with areas of hemorrhage and necrosis.

Microscopy

- Nodular and well-circumscribed
- Nests and cords, may merge into sheets
- Relatively uniform population of intermediate trophoblastic cells.
- Cells contain round, uniform nuclei eosinophilic or clear (glycogen-rich) cytoplasm well-defined cell membrane
- Geographic pattern of necrosis
- Hyaline matrix
- Calcification
- Small blood vessels in center of tumor nests
- The appearance closely simulates that of a carcinoma
- Blood vessels within the tumor are preserved with occasional deposition of amorphous fibrinoid material in the wall.
- A lymphocytic infiltrate often surrounds the tumor.

IHC

- Diffuse reactivity: Keratin, α-inhibin, EMA, p63, HLA-G, and E-cadherin
- Focal reactivity: hPL, hCG, PLAP
- Positivity for **cyclin E** (placental site trophoblastic tumor)
- Negative for p16 (useful in the differential diagnosis with cervical and other types of carcinoma).

Differential Diagnosis

- PSTT
- Placental site nodule
- Choriocarcinoma
- Epithelioid smooth muscle tumor
- Keratinizing squamous cell carcinoma of the cervix.

PLACENTAL SITE NODULE

- Microscopic and paucicellular
- There is no necrosis
- Ki-67 index is markedly low.

Clinical Behavior

- Tend to remain confined with slow growth.
- Metastasis in the lung are most common
- Treatment
  - Combined chemo
  - Hysterectomy
  - Lung resection.

Exaggerated Placental Site

- Benign non-neoplastic lesion
- Excessive but otherwise normal infiltration (into myometrium) of the implantation site by intermediate trophoblast
- Called syncytial endometritis in the past (misnomer).

Pathogenesis

- Identical in morphological and immunophenotypical profile to the implantation site intermediate trophoblastic cells in the normal placental site
- Precise mechanism unclear
- Rapid cell cycle progression of IT in the trophoblastic columns
- Suppression of apoptosis of IT in the implantation site.

Clinical Features

- May be associated with normal pregnancy, abortion, or hydatidiform mole
- Presents with postpartum hemorrhage

Histopathology

- Composed predominantly of mononucleate and variable numbar of multinucleated implantation site intermediate trophoblasts
- Infiltrates endomyometrium but architecture is preserved.
- Glands are surrounded but not destroyed
- Decidua may show degeneration and necrosis
- Villi are seen.

Behavior and Treatment

- Benign
- Involutes following curettage.

Placental Site Nodule

- Placental site nodules (or plaques) are small, well-circumscribed nodular aggregates of chorionic-type intermediate trophoblastic cells
- The cells are embedded in a hyalinized stroma.

Pathogenesis

- Well-circumscribed hyalinized lesion composed of chorionic type intermediate trophoblastic cells
- Believed to represent a portion of an uninvoluted placental site from a remote gestation
- Immunophenotype similar to that of trophoblastic cells in the chorion laeve.

Clinical Features

- Reproductive age group
- Typically incidental findings in uterine or endocervical curettings, cervical biopsies, and occasionally in hysterectomy specimens
- Prior history: Therapeutic abortion, cesarean section and tubal ligation.

Gross

- Small, ranging from 1 to 14 (average 2.1) mm
- Occasionally, multiple and sizable (>5 mm)
- When grossly visible: Yellow-white and necrotic-appearing nodule in the endometrium or superficial myometrium
- Can also occur in the cervix, fallopian tube, and other sites.

Microscopy

- Small nodular or plaque-like lesions with rounded, well-circumscribed borders
- Surrounded by a thin rim of chronic inflammatory cells and, occasionally, decidualized stroma
- Haphazard pattern, dispersed singly, in small clusters and cords, or occasionally diffuse
- Mitotic figures: Absent or rare.
- Cells occupy the outer portion of the nodule with a central hyalinized extracellular matrix
- Cells vary in size; many have relatively small uniform nuclei and a few have large, irregular, and hyperchromatic nuclei
- Multinucleated: Occasionally present
- The cytoplasm of the larger trophoblastic cells is abundant and eosinophilic to amphophilic, whereas the smaller cells contain glycogen-rich clear cytoplasm.

IHC

- Diffusely positive: PLAP
- Focally positive or negative for hPL and CD146 (Mel-CAM).

Atypical Placental Site Nodule

- It shows higher cellularity and greater cohesiveness
- Ki 67: 15–20%.

Differential Diagnosis

- Epithelioid trophoblastic tumor
- Atypical placental site nodules have smaller size
- Better circumscription
- Extensive hyalinization
- Degenerative appearance
- Paucity of mitotic activity
- Absence of cyclin E expression.

Behavior and Treatment

- Benign
- Do not recur or metastasize
- Hysterectomy or curettage
- Patients with β-hCG levels that fail to normalize should be evaluated for GTN.

FIGO Staging of GTN

- Stage I: Disease—localized to the uterus
- Stage II: Disease—localized to the pelvis and adnexa
- Stage III: Pulmonary metastases
- Stage IV: Distant organ involvement (i.e., liver, brain, kidney, GI tract, spleen, etc.)

POINTS TO REMEMBER

- GTDs recapitulate trophoblast present in early developing placenta
- They have different features due to differences in cell from which they are derived
- They can present years after pregnancy.
- Recognition of gestational trophoblastic tumors continues to be problematic, largely because of their relative rarity and significant histologic overlap with common gynecologic tumors.
- However, precise diagnosis of these tumors is crucial for patient management because of their overwhelmingly good response to chemotherapy (gestational choriocarcinoma) or excellent postsurgery outcome.
- Awareness of these rare tumors with a high index of suspicion is crucial for accurate diagnosis.
- Ancillary immunohistochemistry and tissue DNA genotyping may provide valuable and sometimes decisive diagnostic contributions.
- Further refinement of the histologic criteria of early gestational choriocarcinoma and atypical placental site node are needed.

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51 Mesothelial Lesions: Differential Diagnosis and Diagnostic Approach

Poorvi Kapoor

INTRODUCTION

- The mesothelium is composed of simple squamous-like epithelial cells that form the lining of several body cavities like the pleura, peritoneum and the pericardium.
- Mesothelial tissue is also seen surrounding tunica vaginalis of the testis and the tunica serosa of the uterus.

CLASSIFICATION

- Mesothelial lesions can be benign or malignant
- Benign lesions are further classified as:
  - Epithelial
  - Histological patterns seen are as follows:
    - Papillary
    - Adenomatoid
    - Nodular hyperplasia
    - Peritoneal multicystic
    - Mesothelioma
  - Mesenchymal
    - Reactive fibrosis
    - Solitary fibrous tumor
- Malignant lesions are classified as:
  - Epithelioid
  - Patterns seen under this are as follows:
    - Tubular
    - Tubulo-papillary
    - Papillary
    - Solid
    - Adenomatoid
    - Adenoid cystic
    - Pleomorphic
  - Sarcomatoid
  - Patterns seen in sarcomatoid mesothelioma are as follows:
    - Fibrosarcomatous
    - Osteo/chondro/rhabdomyo and leiomyosarcomatous

    - Undifferentiated high-grade pleomorphic sarcoma-like
    - Lymphohistiocytoid
  - Biphasic
  - Desmoplastic.

CLINICAL PRESENTATION

- Pleura
  - Chest pain
  - Breathlessness
  - Heaviness in the chest
  - Dull respiratory sounds
- Pericardium
  - Chest pain
  - Pericardial friction rub
  - Dull heart sounds
  - Cardiac tamponade
- Peritoneum
  - Abdominal distension
  - Difficulty in breathing
  - Intestinal obstruction.

RADIOLOGICAL FINDINGS

- Concentric pleural thickening
- Pleural effusion
- Reduced volume of involved hemithorax
- Tumor nodules within chest wall
- Pleural opacity extending around lung
- Mediastinal lymphadenopathy.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry performed on a cell block plays an extremely crucial role in the differential diagnosis of these lesions. It is summarized in Tables 51.1 to 51.4.

Table 51.1: Differences between benign and malignant mesothelial cells

Feature	*Benign*	*Malignant*
Cell size	20 μ	≥20 μ
Cell configuration	Sheets of polygonal cells	Mulberry-like clusters
Cell shape	Spherical/oval	Bizarre, spindle shaped
Psammoma bodies	Absent	Present
Microvilli	Short, equal length	Long, complex
Cell aggregates	Few, small number of cells	Numerous, large no. of cells
Nucleus	Round to oval, centrally located, even sized, sharply demarcated, slightly granular	Irregular, may be multiple
Chromatin	Delicate	Hyperchromatic, open, vesicular
Nucleoli	Centrally located, readily visible	Large, irregular
Mitosis	Normal	Atypical

Table 51.2: Differences between adenocarcinoma and mesothelioma		
	Adenocarcinoma	*Mesothelioma*
Pattern	Cannon ball	Mulberry
Cells	Columnar	Round/polygonal/cuboidal
Cytoplasm	Delicate, homogeneous	Dense with lacy edges
Cytoplasmic vacuoles	Crisp with round borders	Degenerative
Nucleus	Usually eccentric	Usually central
Pleomorphism	High degree	Lesser
Multinucleated giant cells	Rare	Common
Windows	Unusual	Common

Table 51.3: Immunocytological differences between reactive mesothelial cells and mesothelioma		
	Reactive cells	*Mesothelioma*
Calretinin	+	+
CK 5/6	+	+
p53	–	+
Desmin	+	–
EMA	–	+

Table 51.4: Immunocytochemical differences between adenocarcinoma and mesothelioma			
Stain	*Staining pattern*	*Adenocarcinoma*	*Mesothelioma*
Mucicarmine	C	+	–
CEA	C	+	–
CA 19–9	C	+	–
Leu M1	C + M	+	–
E-cadherin	C + M	+	–
BerEP4	C + M	+	–
B27.3	M	+	–
TTF1	N	+	–
Keratin protein	M (peripheral)	+ (perinuclear)	+
Calretinin	C + N	–	+
Vimentin	C	–	+
WT1	N	–	+

C: Cytoplasmic, M: Membranous N: Nuclear

BENIGN EPITHELIAL MESOTHELIAL LESIONS

- Well-differentiated papillary mesothelioma
- Adenomatoid tumor
- Multicystic mesothelioma
- Nodular multicystic mesothelial histiocytic hyperplasia

Well-Differentiated Papillary Mesothelioma

- It is seen mainly in the peritoneum and shows a predisposition for women between 30–40 years
- They present with pain and serous effusion
- Macroscopically, there is a multifocal, vegetative proliferation
- Microscopically, papillae are seen with a fibrovascular stalk lined by mesothelial cells
- There is no mitosis, but psammoma bodies may be seen.

Differential Diagnosis

- Non-specific reactive mesothelial hyperplasia (no mass formed)
- Epithelial malignant mesothelioma with predominant papillary pattern
- Serous papillary carcinoma of the ovary and peritoneum (mesothelial markers are negative).

ADENOMATOID TUMOR

- It is seen in the mesothelium of para-testicular region and serosal surface of the uterine wall.
- It can also be seen in ovary, salpinx and broad ligament and rarely in the pleura and pericardium.
- Microscopically, cords of cuboidal to flattened cells that form channels with dilated lumina simulating vessels.
- Cells have acidophilic cytoplasm with vacuoles.
- There are no nucleoli, mitoses or necrosis.
- Surrounding stroma shows desmoplastic response with elastic fibers and smooth muscles along with inflammatory cells.
- Adenomatoid, angiomatoid, solid and cystic patterns can be seen.

PERITONEAL MULTICYSTIC MESOTHELIOMA

- It is almost exclusively seen in the peritoneum and may exceptionally be seen in the testis or pleura.
- It occurs in young females who present with palpable abdominal mass and pain.
- Grossly, it is seen as a well circumscribed mass comprising several cysts with thin walls and variable size containing serous fluid.
- Microscopically, cystic spaces are seen lined by a single layer of bland cuboidal mesothelial cells filled with eosinophilic serous fluid.
- There are no nucleoli or mitosis and surrounding stroma is hypocellular.
- Immunohistochemistry shows positivity for cytokeratin, calretinin and CD31 and CD34 negativity.
- Differential diagnosis is multicystic lymphangioma.

NODULAR HISTIOCYTIC/MESOTHELIAL HYPERPLASIA

- Within reactive hyperplastic changes, a solid pattern may be focally or extensively present.
- These nests of cells are often seen during cardiac surgery as excrescences.

- It is characterized by nests or nodular aggregates of round mesothelioid cells which are oval to polygonal with eosinophilic cytoplasm and small, eccentric, round to oval nucleus.
- These cells are positive for CD68 and cytokeratin.

BENIGN MESENCHYMAL MESOTHELIAL LESIONS

- Pleural plaques
- Chronic fibrous pleurisy
- Solitary fibrous tumor.

Pleural Plaques

- They are firm, sometimes calcified lesions present in the parietal pleura and known to be associated with asbestos exposure.
- Grossly, they are firm and characterized by calcification.
- Microscopically, they are characterized by hypocellular collagen rich mesenchymal proliferation with a distinctive basket-weave pattern.

Chronic Fibrous Pleurisy

- Microscopically, they show a zonal pattern ranging from active inflammation to quiescent dense fibrosis along with fibrin and granulation tissue.
- The features of malignancy are absent, i.e. there is no atypical mitosis or invasion of surrounding tissues.
- A differential diagnosis of sarcomatous mesotheliomas may be considered, but these show consistent spindle cell proliferation.
- Desmoplastic malignant mesothelioma should also be ruled out. These lesions are paucicellular, sclerotic and have bland necrosis, which is typical for these lesions.

Solitary Fibrous Tumor

- They are located mainly in the pleura, can occur at any age and do not show any sex predilection
- It is usually asymptomatic and an incidental finding and is not associated with asbestos exposure
- They are known to be associated with paraneoplastic syndromes like migrating thrombitis, thrombocytosis, hemolytic anemia, hypoglycemia and hypercalcemia.
- Grossly, they are pedunculated, polypoidal masses and can reach up to 40 cm in size. On cut, they are firm and fibrous.
- Microscopically, several features have been described, including hemangiopericytomatous, sclerosing and myxoid.
- These tumors are positive for CD34.

MALIGNANT MESOTHELIAL LESIONS: MESOTHELIOMA

Malignant mesothelioma can be localized or diffuse.

- **Localized malignant mesothelioma:**
  - 20% of primary malignant pleural tumors are localized
  - They present as symptomatic masses and are difficult to differentiate from chest wall neoplasms.

- **Diffuse malignant mesotheliomas:** It is a classical form which is related to asbestos exposure with a latency period of around 20 years.

Malignant Mesothelioma Comprises Three Histological Types

1. **Epithelioid:**
   - It is seen in 50% cases and most often confused with adenocarcinomas
   - The cells are polygonal, oval or cuboidal and often mimic reactive mesothelial cells.
2. **Sarcomatoid:**
   - It is seen in 16% cases
   - Spindle cells are usually seen with elongated nuclei, numerous mitotic figures and eosinophilic cytoplasm
   - But it can also be composed of lymphohistiocytoid cells and may contain heterologous mesenchymal elements.
3. **Mixed:**
   - It is seen in 34% of cases and contains both epithelioid and sarcomatoid areas
   - These areas usually blend with one another showing transitional areas
   - It can be differentiated from synovial sarcoma by the absence of intracellular PAS positive mucin.

- Grossly, malignant mesotheliomas usually present as confluent nodules or form a diffuse thick rind-like growth that encases the subjacent organs. It can rarely manifest as a sharply circumscribed serosal tumor.
- A list of histological types of malignant mesothelioma presented in Table 51.5.

Table 51.5: Histological types of malignant mesothelioma

Epithelial	*Sarcomatoid*
Tubulopapillary	Fibrosarcomatoid
Solid	Chondrosarcomatoid
Adenomatoid	Osteosarcomatoid
Small cell	Leiomyosarcomatoid
Clear cell	Malignant fibrous histiocytoma like
Deciduoid	Lymphohistiocytoid
Adenoid cystic	Desmoplastic
Pleomorphic	Biphasic

Differential Diagnosis of Epithelioid Malignant Mesothelioma

Reactive mesothelial proliferation

- They are cellular, confined to the mesothelial surface and not in the stroma
- They show simple papillae lined by a single layer of cells unlike malignant lesions which show complex papillae, tubules and stratification
- There is no stromal invasion and necrosis is rare
- There is uniformity of growth and inflammation is commonly seen
- EMA, GLUT-1, p53 and IMP3 are negative (whereas malignant lesions are positive for these markers)
- Desmin is positive

- Metastatic deposits from carcinoma lung, breast, kidney or liver
- They are PAS positive and diastase resistant and also show positivity on staining with Alcian Blue.

Immunohistochemistry

- International Mesothelioma Interest Group (IMIG) recommends two mesothelial and two carcinoma markers along with pancytokeratin.
- Best positive mesothelial markers are Calretinin, CK5/6, WT-1 and D2-40
- Best positive carcinoma markers are MOC-31, BerEP4, CEA and BG8
- Best positive lung adenocarcinoma markers are TTF1 and Napsin.
- Electron microscopy helps differentiate mesotheliomas from adenocarcinomas (Table 51.6)

Table 51.6: Ultrastructural differences between mesotheliuom and adenocarcinoma

Feature	*Mesothelioma*	*Adenocarcinoma*
Apical microvilli	Long and thin, no glycocalyx	Shorter, have microvilli
Perinuclear tonofilament bundles	Present	Absent
Basal lamina	Present	Absent
Long desmosomes	Present	Absent

Differential Diagnosis of Sarcomatous Malignant Mesotheliomas

- Fibrous pleurisy
- Benign and malignant solitary fibrous tumor
- Sarcomas
  - CD31, CD34, desmin, myoglobin, S100, seen in different types of sarcomas are negative in mesotheliomas
  - D2-40, Cam5.2, WT1, AE1/AE3 markers are positive in mesotheliomas
- Melanoma
- Synovial sarcoma: which can be tested for its distinctive translocation t(X;18)
- Sarcomatoid renal cell carcinoma.

Molecular markers in Malignant Mesothelioma

- The homozygous deletion of 9p21 is the most common genetic alteration
- BRCA associated protein 1 (BAP1) germline mutations can identify those at increased risk
- NF2 inactivating mutations are also seen along with mutations in the LATS2 gene
- p16/CDKN2A deletions are seen in nearly 80% of primary pleural mesotheliomas with nearly all cases of sarcomatoid type showing this deletion. In contrast, only 25% peritoneal mesotheliomas.

POINTS TO REMEMBER

- The diagnostic process of mesothelial lesions is complex.
- It can be one of the greatest challenges faced by the practicing surgical pathologist
- Evidence of malignant mesothelioma on cytological examination should be confirmed with histological analysis, or if biopsy is not feasible, the cytological diagnosis should be always supported by clinical, radiologic, and surgical findings.

- The identification of the histological appearance (epithelioid, biphasic, sarcomatoid) of malignant mesothelioma could facilitate diagnosis and provide important prognostic information since the histotype is still the best predictor of prognosis.
- IHC is fundamental for the diagnosis and differential diagnosis of malignant mesothelioma.
- The immunohistochemical approach should rely on the application of a panel including positive (mesothelial-related) and negative markers as suggested by morphology and clinical information when available.

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52 Molecular Pathogenesis of Lung Cancer

Harsh Batra

INTRODUCTION

- According to GLOBOCAN 2018 survey, lung cancer contributes to largest number of newly diagnosed cases of cancer.
- Number of deaths in cancer patients accounting to 1.8 million deaths globally.
- Lung cancer is the most common cancer in men worldwide.
- Although it is the seventh most common cancer in women, it has surpassed breast cancer in terms of mortality.
- Adenocarcinoma is the more common histological subtype.

Risk Factors

- Majority of the cases occur due to tobacco smoking (80–90%).
- Cigarette smokers have a tenfold or greater increase in risk.
- One genetic mutation is induced for every 15 cigarettes smoked.
- In Indian patients with lung cancer, history of active tobacco smoking was found in 87% of males and 85% of females.
- History of passive tobacco exposure is found in 3% cases in India.
- A smaller proportion (10–20%) is attributable to occupational exposure to various carcinogenic agents.
- Asbestos exposure, nickel mining, ionizing radiations, chromium, hematite mining causing radon exposure are a few of the associated risk factors
- Increased rates of lung cancer among survivors of the atom bombs dropped on Hiroshima and Nagasaki have been found.
- Plant carotenoids and lycopene seem to have a protective role against lung cancer.
- Studies have shown that coal smoke which is generated as a domestic pollutant fuel contains potential carcinogens viz. SO_2, CO TSP, radon and thoron.

Genetics of Lung Cancer

- Cytogenetic studies have identified many chromosomal alterations in lung cancer.
- Mutations include activation of the dominant cellular proto-oncogenes (which promote oncogenesis) of the RAS and MYC family and inactivation of the recessive or tumor suppressor genes.
- Genetic instability in lung cancer can occur at multiple levels which include:
  - Chromosomal level—aneuploidy, deletions and non-reciprocal alterations
  - Microsatellite alteration—35% of SCLC and 22% of NSCLC

- Mutation in OGG1 and inactivation of DNA repair gene O6-methylguanine methyl transferase by promoter hypermethylation.

- Small cell lung cancer (SCLC) is associated with oncogenes like c-myc, L-myc, N-myc, c-raf and tumor suppressor genes like p53 and Rb.
- Non-small cell lung cancer (NSCLC) is associated with K-ras, N-ras, H-ras, c-myc, c-raf and tumor suppressor genes like p16 and Rb.

MOLECULAR MARKERS IN LUNG CARCINOMA

RAS

- The RAS proto-oncogene family includes KRAS, HRAS, and NRAS.
- KRAS mutation is the most frequent in lung cancer, mutations at codon 12, codon 13 and codon 61.
- KRAS mutations affect approximately 20% to 30% of lung adenocarcinomas and 15% to 20% of all NSCLCs but rarely SCLCs.
- It is associated with smoking and a weak negative prognostic indicator.
- RAS mutation may be associated with resistance to EGFR TKI (tyrosine kinase inhibitor) therapy.
- Reolysin, a formulation of reovirus, and FusOn-H2 are currently in clinical trials or under development for the treatment of various cancers.

EGFR

- Gene for EGFR lies on chromosome 7p11.2, 200 kb, and contains 28 exons, encodes a protein of 464 amino acids.
- EGFR is composed of an N-terminal extracellular ligand-binding domain, a transmembrane lipophilic segment, and a C-terminal intracellular region containing a tyrosine kinase domain.
- EGFR mutations are responsible for the constitutive activation of tyrosine kinase receptor.
- In adenocarcinomas, majority of mutations have been identified in exons 18–21 of EGFR gene.
- These mutations can be roughly classified into three major categories:
  - In-frame deletions in exon 19,
  - Insertion mutations in exon 20, and
  - Missense mutations in exons 18–21.

EGFRvIII

- EGFR variant III (EGFRvIII) is a mutation resulting from an in-frame deletion of exons 2–7 of the coding sequence (amino acids 6–273).
- It has been associated with a subset of squamous cell lung cancers.
- EGFRvIII has also been identified in glioblastoma, breast cancer, ovarian cancer, prostate cancer, and lung cancer.
- Although EGFRvIII fails to bind EGF, its intracellular tyrosine kinase is constitutively activated, allowing the receptor to undergo tyrosine autophosphorylation.
- EGFRvIII activates phosphatidylinositol 3′ kinase (PIK3CA) signaling pathway, which provides critical information for cell survival, proliferation, and motility.

- EGFR mutations are primarily seen in adenocarcinoma.
- Therefore, EGFR mutation analysis is the best predictive marker for the use of EGFR-TKI therapy in NSCLC with an adenocarcinoma component.
- The most common mechanism of resistance to EGFR-TKI is the T790M gatekeeper mutation, caused by a single-base substitution of C to T, at nucleotide 2369.

ALK

- The ALK gene located at chromosome 2p23.2 encodes a receptor tyrosine kinase.
- It is found in several fusion proteins consisting of intracellular kinase domain of ALK and the amino-terminal portions of different genes.
- EML4–ALK fusion is formed as the result of a small inversion within the short arm of chromosome 2 t.
- It joins intron 13 of echinoderm microtubule-associated protein-like 4 (EML4) to intron 19 of ALK [inv(2)(p21;p23)].
- This generates an oncogenic fusion encoding a constitutively activated protein tyrosine kinase.
- EML4–ALK fusion is a rare abnormality detected in ~3–13% of patients with adenocarcinomas.
- With at least seven EML4-ALK variants known, the most common fusion results from the joining of exons 1–13 of EML4 to exons 20–29 of ALK.
- The key downstream effectors of the ALK pathway include the RAS-activated protein, extracellular signal-regulated kinase (MAPK1), phosphoinositide 3-kinase (PIK3CA), and STAT3 signaling pathways.
- RAS/MAP2K1/MAPK1 pathways are critical for cell proliferation, whereas the PIK3CA/AKT1 and STAT3 pathways are important for cell survival.
- Patients with the EML4–ALK fusion gene tend to be:
  - Younger (median, 52 vs 64 years)
  - Diagnosed at an advanced clinical stage at presentation
  - Male dominant (58 vs 32%)
  - More likely to be never-smokers (74 vs 26%).
- The United States Food and Drug Administration approved the ALK Break Apart fluorescence in situ hybridization (FISH) Probe Kit (Abbott Molecular, Des Plaines, IL, USA) as a companion diagnostic for targeted therapy with Crizotinib in lung cancers.

MYC

- MYC heterodimerization transcriptionally activates downstream genes, which drives cells to grow.
- Activation occurs by gene amplification (~20–115 copies per cell) or by transcriptional dysregulation, both of which lead to protein overexpression.
- 18% to 31% of SCLCs had amplification of one MYC family member.
- Conversely, only 8% to 20% of NSCLCs were affected.
- MYC amplification appears to occur more frequently in chemotherapy-treated patients and the "variant" SCLC subtype.
- It may correlate with adverse survival.

P53

- Its chromosome 17p13 locus is frequently hemizygously deleted,
  - Mutational inactivation of the remaining allele occurs in 75% or more of SCLCs and about 50% of NSCLCs.
- p53 mutation is associated with cigarette smoking.
- Abnormal p53 expression by immunohistochemistry in 40% to 70% of SCLCs and 40% to 60% of NSCLCs (squamous cell carcinomas higher than adenocarcinomas).
- Prognostic value of p53 is controversial but mutations have been linked to response to cisplatin-based chemotherapy in NSCLC and response to radiotherapy.
- Presence of antibodies against p53 have been seen in 15–25% of cases.
- Their prognostic value has been controversial.
- Nonetheless, they seem to be reduced during chemotherapy.
- p21 is a p53-responsive gene that inhibits cyclin/cyclin-dependent kinase (CDK) complexes at the G1 phase.
- p21 was overexpressed in 65% to 75% of NSCLCs.
- The MDM2 oncogene product inhibits p53 function.
- MDM2 protein is overexpressed in 25% of NSCLCs.
- MDM2expression without abnormal p53 expression (mutation) is a favorable prognostic factor.

p16-cyclin D1-CDK4-RB Pathway

- Inactivation of both RB alleles at chromosome region 13ql4 is common in lung cancers, with protein abnormalities detected in about 90% of SCLCs and 15% to 30% of NSCLCs.
- Functional RB loss can include deletion, nonsense mutations, or splicing abnormalities, frequently leading to a truncated RB protein.
- Whether absent RB expression is associated with poor prognosis in NSCLCs is controversial.
- Functionally, *in vitro* reintroduction into tumor cells of a wild-type RB suppresses SCLC growth
- Two RB-related genes also have been implicated in lung cancer including, p107 and pRB2/p130.
- Decreased expression of these proteins is associated with more aggressive clinical behavior.
- Cyclin D1 is overexpressed in 25% to 47% of primary NSCLCs and in some cases has been associated with poor prognosis.
- Transfection of a cyclin D1 antisense construct into lung cancer cell lines (and thus inhibiting any tumor-related cyclin D1 production) induces destabilization of RB and retards growth.
- A CDK inhibitor, flavopiridol, is in clinical trials against lung and other cancers.

p16

- p16 abnormalities are frequent in NSCLC but rare in SCLC.
- Hypermethylation in the 5´CpG island of retained allele may also cause loss of expression of pl6 from the remaining allele in lung cancer.

- p16 alteration and loss of function may be associated with tumor progression, clinical stage, and survival.
- The p16 locus also encodes a second alternative reading frame protein, p19ARF, that overlaps with p16.
- p19ARF protein expression was more frequently lost in tumors with neuroendocrine features.

Nicotine and Opioid Receptor

- Lung cancer cells express nicotine receptors and a variety of opioid receptors, as well as being able to produce several opioid peptides.
- Opioids can inhibit lung cancer cell growth and cause apoptosis.
- Nicotine acting through specific, high-affinity, nicotinic acetylcholine receptors on lung cancer cells can antagonize the apoptotic effect of opioids.
- Thus it is possible that inhaled nicotine from smoking may play a role in lung cancer pathogenesis by inhibiting apoptosis, for example, in precursor lesions.

PTEN

- Protein tyrosine phosphatases antagonize the growth-promoting protein kinases.
- Phosphatase is encoded by the PTEN gene at chromosome 10q23.
- PTEN is mutated in a few primary lung cancers, and several lung cancer cell lines show homozygous deletions of this gene.

HER2/neu

- HER2/neu is highly expressed in approximately 30% of NSCLCs, especially adenocarcinomas.
- On ligand binding, ERBB receptors homodimerize or heterodimerize, thereby inducing intrinsic kinase activities that initiate intracellular signal transduction cascades including the MAP kinase pathway.
- High ERBB2 levels are associated with the multiple drug resistance phenotype and increased metastatic potential in NSCLC.
- HER2- mutated adenocarcinoma cases have been reported to respond to trastuzumab and afatinib.

Apoptotic and Anti-apoptotic Genes Involved in Evasion of Apoptosis

- Expression of the Bcl-2 is higher in squamous cell carcinoma (25–35%) than in adenocarcinoma (~10%) and correlates with neuroendocrine differentiation and is higher in SCLCs (75–95%) than in NSCLCs.
- BAX, which counteracts Bcl-2 and promotes apoptosis, is a downstream transcription target of p53.
- Expression of BAX and Bcl-2 are inversely related in neuroendocrine cancers.
- Bcl-2 antisense in SCLC inhibits the translation of Bcl2 mRNA and down-regulates Bcl-2 protein expression.
- Bcl-xL antisense in NSCLC. Bcl-2-BclxL antisense target both SCLC and NSCLC.
- Fas (CD95) and Fas ligand (FasL) also play key roles in the initiation of apoptotic pathways.

- Lung cancer cell lines and tumors express FasL, and co-culture of lung cancer lines with a Fas-sensitive human T cell line induces T cell apoptosis.
- Fas (CD95) and Fas ligand (FasL) also play key roles in the initiation of apoptotic pathways.
- Lung cancers can make FasL but cannot respond to it because they lack the receptor, Fas.
- FasL inactivates T cells, providing the mechanism of escape from patient's immune response.

Discoidin Domain Receptor 2 (DDR2)

- DDR2 encodes a membrane-bound receptor tyrosine kinase that binds collagen and is involved in the regulation of cell proliferation and survival.
- Mutations of DDR2 gene occur in 3.8% of squamous cell carcinomas and are associated with oncogenic activity that may respond to Dasatinib.

FGFR1

- FGFR1 is a membrane-bound tyrosine-kinase receptor involved in the regulation of cell proliferation and angiogenesis through activation of the MAPK and PI3K pathways.
- FGFR1 amplification occurs more frequently in squamous cell carcinoma (21%) than in adenocarcinoma (3%).
- The novel FGFR inhibitor ponatinib may be effective in patients with FGFR1 overexpression and the results of ongoing trials are pending.
- Other genes involved are shown in Table 52.1.

Table 52.1: Other genes involved in lung cancer

Gene	*Adenocarcinoma*	*SCC*	*IHC*	*FISH*	*Other*
ROS1	2% (translocation)	Nil	±	+	–
RET	2% (translocation)	Nil	–	+	–
BRAF	2% (mutation)	Nil	±	–	+
MET	5% (amplified)	10% (amplified)	±	+	–

Epigenetic Modification in Lung Cancer

- Epigenetic modifications refer to several molecular mechanisms that regulate gene expression without changing the DNA sequence. These include the following:
- Alteration of the methylation status of DNA within CpG islands, with hypermethylation of CpG island promoters of tumor suppressor genes leading to their silencing.
  - Covalent modification of histone tails.
- Gene regulation by micro-RNA (miRNA).

MOLECULAR TESTING OF LUNG CARCINOMA

- EGFR mutation testing and ALK rearrangement status are the only two molecular markers considered the standard-of-care of NSCLC treatment in daily practice currently.

- **Histologic subtyping:** It has become clinically relevant to distinguish between adenocarcinoma and squamous cell carcinoma, as the chemotherapy regimen differs for these histologic subtypes.
- Pathologists should try to classify further poorly differentiated NSCLC with the application of a panel of immunohistochemical markers, such as thyroid transcription factor-1 and napsin A to identify an adenocarcinoma component.
- p63/p40 is used to identify a squamous cell carcinoma component.
- **Molecular testing guidelines:** Adenocarcinoma is the basis for conducting molecular testing.
- For limited specimens, EGFR testing should be prioritized first and ALK testing should be prioritized second over other molecular markers.
- In early-stage cancer both EGFR and ALK testing is encouraged.
- Techniques such as direct sequencing, pyrosequencing, and peptide nucleic acid polymerase chain reaction (PCR) clamping, are recommended for EGFR mutation testing.
- FISH assays using dual-labeled break-apart probes are recommended for ALK gene rearrangement.
- Next generation sequencing and Multiplex PCR [can evaluate "hot spot" mutation; e.g. SNaPshot (Applied Biosystems, Foster City, CA, USA) and Sequenome Mass ARRAY (Sequenom Inc., San Diego, CA, USA)] are promising technologies which hold great prospects in future.

The Cancer Genome Atlas (TCGA) Project

- The TCGA Network plans have achieved the ambitious goal of analyzing the genomic, epigenomic, and gene expression profiles of more than 10,000 specimens from 25 different tumor types including lung cancer.
- TCGA has already identified both common and unique mutation spectra and pathway activation in 183 lung adenocarcinomas and 178 squamous cell carcinomas.
- Two major histologies in NSCLC.
- In adenocarcinoma 25 genes showed statistically significant mutation. One of the most significantly mutated genes in this lung adenocarcinoma cohort was U2AF1, which had non-synonymous mutations in 3% of cases. U2AF1 mutation may confer tumorigenic capability independent of known proliferations-sustaining driver genes.
- Lung squamous cell carcinoma is characterized by complex genomic alterations.
- In total, 18 genes were shown to have a statistically significant number of mutations including mutation of TP53 in nearly all specimens.

POINTS TO REMEMBER

- Lung cancer is the leading cause of cancer-related death worldwide due to late diagnoses and limited treatment interventions.
- Recently, comprehensive molecular profiles of lung cancer have been identified.
- These novel characteristics have enhanced the understanding of molecular pathology of lung cancer.
- The identification of driver genetic alterations and potential molecular targets has resulted in molecular-targeted therapies for an increasing number of lung cancer patients.

- Thus, the histopathological classification of lung cancer was modified by the increased understanding of molecular profiles.
- In the present era we have comprehensive genomic data of lung cancer.
- Thus effort should be made to treat lung cancer through more efficacious targeted therapeutic interventions.

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53 Notochord Tumors: Newer Concepts

INTRODUCTION

- The notochord forms after the third gestational week of development.
- It has a role in inducing the formation of the neural plate and vertebral column.
- It also provides structural support for the development of the axial skeleton.
- During development, the notochord regresses in the region of the vertebral bodies and intervertebral discs to eventually disappear.
- It remains as the nucleus pulposus.
- Notochordal remnants have occasionally been found in the intervertebral disk called rests or vestiges.
- These usually disappear by the age of 1 to 3 years.
- However, rarely do they persist into adulthood.

ECCHORDOSIS/ ECCHORDOSIS PHYSALIPHORA

- These are remnants of fetal notochord with limited growth.
- Rarely, it persists in adults in the following sites:
  - Nucleus pulposus of intervertebral disc.
  - In vertebral body as a benign notochordal cell tumor.

History

- It was first described by ***Luschka*** in 1856 and named ***ecchondrosis/ecchordosis physaliphora spheno-occipitalis.***
- In an autopsy series published in 1894 it was found in ***2% of autopsy cases,*** and the concept of a notochordal origin was introduced.
- In another autopsy series in 1923 of 200 cases extravertebral notochordal, rests were found in ***1.5% of the autopsies.***
- In 1982 the name of ecchordosis physaliphora ***vertebralis*** was given to this entity.

Epidemiology

- It has been identified in 6 of 1600 autopsies with an overall incidence of 0.38%.
- There is a wide age distribution.
- The common sites are along the midline of craniospinal axis and in the intracranial region retroclival.

Clinical Presentation

- These cases are generally asymptomatic.
- Usually diagnosis is made on CT obtained for nonspecific symptoms, such as headache.
- Very rarely, patients may have fatal pontine hemorrhage or CSF fistula.

Radiology

- Bony destruction is not seen.
- No enhancement seen even after contrast.
- Osseous stalk seen arising from clivus is often present on CT.
- The osseous stalk is considered hallmark of ecchordosis.

Morphology

- **Gross:** Well-circumscribed, round to oval, gelatinous nodule. The size usually ranges from a few millimeters to 2 cm.
- **Microscopy:**
  - Large cells arranged in cords or clusters are seen.
  - Varying amounts of basophilic myxoid matrix.
  - The cells show abundant eosinophilic to clear multivacuolated cytoplasm.
  - Nuclei are round to oval.
  - No significant nuclear atypia, mitotic activity, or necrosis are seen.

BENIGN NOTOCHORDAL CELL TUMOR (BNCT)

- Previously known as ***giant notochordal cell rest/notochordal hamartoma.***
- It is a distinct slow-growing notochordal cell proliferation.
- Usually behaves in an indolent fashion.
- Benign notochordal cell tumors are usually incidental microscopic findings found on autopsy.
- **Location:** The usual location is the axial skeleton with common sites being sacrum, coccyx, cervical and lumbar vertebrae.
- **Clinical presentation:** They may be seen at various ages and in both sexes.
- They are usually asymptomatic.
- Very often they are an incidental finding during work-up for trauma, neck stiffness from degenerative joint disease, or following a fall or a motor vehicle accident.
- When symptomatic, the most common complaint is back pain
- The pain can be chronic or acute onset of a few months.
- Depending on the location of the lesion following clinical features may be seen:
  - Limitation of movement.
  - Numbness.
  - Pain of fingers, neck, upper back, buttock.
  - Shoulder stiffness.

Radiological Findings

- The smaller BNCTs are not seen radiologically because they are incidental microscopic findings.
- Computed tomography often shows a sclerotic lesion.

- MRI on T2-weighted images shows homogenous high-signal intensity (bright), whereas T1-weighted images are homogenous low-signal intensity.
- The tumors are well-circumscribed, involving their respective vertebrae without extension or destruction beyond the vertebra.

Morphology

- **Gross :** They are usually small tan-white and gritty in consistency.
- They are well-delineated tumors filling up the vertebra.
- The surrounding bone is intact.
- No gelatinous areas or visible bone destruction are seen.
- **Microscopy:**
  - These are well-delineated lesions.
  - The intertrabecular spaces are filled by nests or sheets of clear cells that resemble adipocytes.
  - They have clear cytoplasm and eccentrically located, round, or oval nuclei.
  - Cells containing multiple clear, cytoplasmic vacuoles, indenting the nucleus, known as ***physaliphorous cells*** are seen.
  - Occasionally, these cells have a pink cytoplasm with eosinophilic globules of varying sizes.
  - These globules are PAS(+) and diastase resistant.
- **Differential diagnosis:** The main differential diagnosis is with chordoma. It is differentiated from chordoma by the absence of the following features:
  - Myxoid stroma
  - Mitoses
  - Nuclear pleomorphism
  - Necrosis.

Immunohistochemistry

- The tumor is strongly cytokeratin positive.
- Immunoreactive to EMA, S100, and vimentin.
- Brachyury is a very sensitive and specific marker for notochordal lesions.
- However, it does not differentiate amongst the notochordal tumors.
- ***The important thing to note is the low Ki-67 (MIB-1) proliferation index which is usually <1%.***

CHORDOMA

- Chordoma is a rare malignant primitive bone tumor.
- It arises from fetal notochord, usually within vertebral bodies.
- Chordoma is of following three types:
  1. Conventional chordoma
  2. Chondroid chordoma
  3. Dedifferentiated chordoma

History

- Virchow (1857) originally described chordomas when he named them ecchondrosis physaliphora, believing they were of cartilaginous origin.

- Ribbert (1895) pierced a nucleus pulposus and found similar tumorous. From this bit of evidence, he correctly proposed the notochordal origin of these tumors.

Etiopathogenesis

- BNCT is thought to be a precursor lesion to chordoma.
- However, it may also arise directly from the notochordal remnant without an intervening stage of BNCT.
- Familial tumors have been associated with the upregulation of brachyury (nuclear transcription factor).
- Brachyury is believed to be a crucial regulator of chordomagenesis.
- Increased incidence in patients with tuberous sclerosis.

Location

- Sacrococcygeal (40–59 years) region: 50%.
- Spheno-occipital/clivus (particularly children): 35%.
- Thoracolumbar spine: 15%.

Incidence

- As primary intracranial neoplasms, they constitute 0.2% of all CNS tumors.
- Among the primary bone neoplasm, they form 2–5% of the cases.

Clinical Features

- These tumors usually occur in males (M: F ratio is 2:1).
- The common age of occurrence is 4th–8th decade.
- Only 5% of tumors occur in those who are less than 20 years of age.
- In children, they usually arise from the skull base.
- They are slow growing tumors with repeated recurrences.
- The symptoms depend on the site and include the following:
  - Tumors in the sacrum lead to back pain, autonomic symptoms like rectal dysfunction or urinary incontinence, or a palpable sacral mass.
  - Intracranial tumors present with features like diplopia, headache, cranial nerve palsies, CSF rhinorrhea, nasal obstruction, nasal bleeding, and subarachnoid hemorrhage.
  - If located in the cervical vertebrae, then they cause hoarseness of voice and dysphagia.

Radiological Picture

- It presents as a destructive lytic tumor that often extends as a soft tissue mass.
- In the sacrum the tumor is characteristically located anteriorly and may displace rectum and extends along sacral nerve roots into the sciatic notch.
- CT shows a radiolucent mass along with bone destruction and calcification.
- MRI on contrast-enhanced T1 weighed image shows some degree of enhancement due to proliferation of vessels.
- MRI-T2 shows a high-intensity lobulated pattern.

Gross

- They appear as irregular lesions that destroy the bone and extend to surrounding soft tissue structures.
- Soft in consistency and grayish tan in color (Fig. 53.1).
- They have an oozing, mucogelatinous appearance.
- They appear as a lobulated mass with size varying from small tumors (2–5 cm) at skull base to large (>10 cm) at the sacrum.

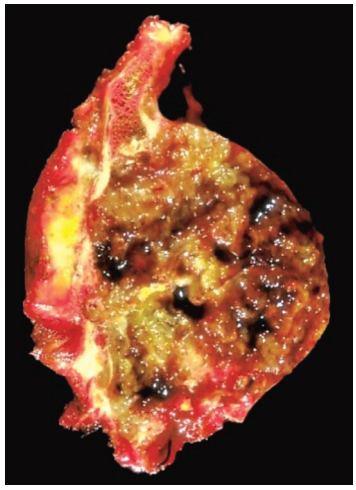

Fig. 53.1: Chordoma in sacrum showing grayish tan, mucogelatinous appearance with soft tissue extension

Microscopy

- There are three types:
  - Conventional chordoma
  - Chondroid chordoma
  - Dedifferentiated chordoma
- In the conventional type tumor shows a distinct lobularity with fibroblast-rich fibrous septae (Fig. 53.2).
- Intercellular myxoid matrix is seen.
- Classic ***physaliphorous cells*** are seen either individually or forming irregular cords (Fig. 53.2).
- The cells show pale and bubbly cytoplasm with fine, intracytoplasmic septations or abundant eosinophilic cytoplasm with fewer than absent intracytoplasmic bubbles.
- Chordoma needs to be differentiated from benign notochordal cell tumor. The differentiating features are as follows:
  - Pleomorphism
  - Moderate to severe nuclear atypia
  - Mitotic figures
  - Destruction of bone trabeculae
  - Necrosis.
- The above-mentioned features are not seen in BNCT.

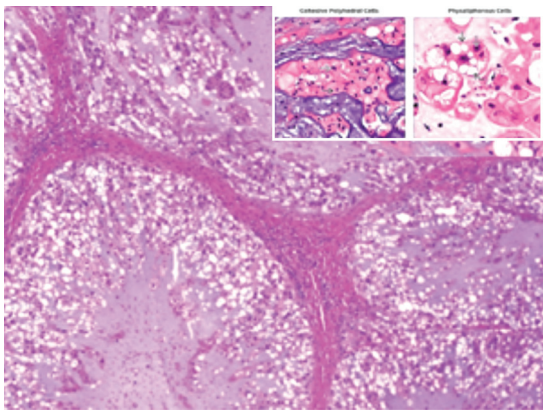

Fig. 53.2: Conventional chordoma showing a classic lobulated pattern on low magnification. Inset in higher magnification shows the characteristic classical physalipharous cells with pale bubbly cytoplasm

Immunohistochemistry

- The tumor cells express epithelial markers like cytokeratin and EMA.
- T brachyury (nuclear transcription factor)—positive.
- S100 protein—positive.
- Vimentin—variably positive.
- CEA and GFAP—variably positive.

Chondroid Chordoma

- It is a tumor with areas of conventional chordoma and regions resembling low-grade, hyaline-type chondrosarcoma.
- It usually arises in the skull base.
- Less frequently seen sites are spine and sacrococcygeal region.

Microscopy

- Chondroid regions merge with or abruptly abut surrounding conventional component.
- Neoplastic cells are surrounded by solid-appearing hyalinized matrix similar in appearance to hyaline cartilage.
- Quantity of chondroid component is variable.
- In some tumors, chondroid areas are so abundant that it may be difficult to distinguish the tumor from chondrosarcoma.

Dedifferentiated Chordoma

- Rarest subtype constituting <5% of all conventional chordomas.
- Dedifferentiation results from ongoing cumulative mutations in conventional chordoma cells.
- May arise *de novo* from primary tumor or as a component of recurrence.
- It may also be within a tumor that has been previously irradiated.
- This particular variant is highly aggressive and has a poor prognosis.

Microscopy

- It is composed of a high-grade sarcoma which is juxtaposed with a conventional chordoma.
- The sarcomatous component is usually a high-grade, undifferentiated pleomorphic sarcoma.

BNCT VS. Chordoma

WHO defining criteria: By imaging

BNCT	*Chordoma*
Subtle hazy sclerosis	Cortical extension
No bony destruction	Aggressive bone destruction
No soft tissue extension	Soft tissue mass
No enhancement with gadolinium	Some degree of enhancement

WHO criteria: By histology

Features	*BNCT*	*Chordoma*
Lobulation	Absent	Present
Cording of cells	Absent	Present
Fibrous septae	Absent	Present
Myxoid matrix	Absent	Present
Necrosis	Absent	Present
Mitosis	Absent	Present
Significant atypia	Absent	Present

ATYPICAL NOTOCHORDAL CELL TUMORS

- These are a series of notochordal-derived tumors that defy current classification schemes.
- Many of these tumors had characteristic histologic features of BNCT, with the exception of minimal soft tissue extension.
- So, a provisional designation of atypical notochordal cell tumors (ANCT) has been proposed for this subset of notochordal-derived tumors.
- They fail to fulfil current diagnostic criteria for either BNCT or chordoma and show presence of atypical radiologic and/or histologic features.

Importance of Diagnosis

- The data suggest that irrespective of whether ANCT eventually progresses to chordoma, there may be a significant time interval wherein no (or minimal) disease progression occurs.
- In a clinical setting, the risk of neurological deficit associated with en bloc oncologic vertebrectomy or sacrectomy may not be warranted.
- Thus, a conservative approach using a careful clinical follow-up with frequent serial imaging may be appropriate in these cases.
- The limitation of small number of cases is also responsible for confusion regarding the biological behavior of these cases.
- With additional cases and longer clinical follow-up it may be possible to designate these cases as benign, intermediate or malignant notochordal neoplasm.

Prognosis

- **Age and gender:** The tumor has a poor outcome in those with a large tumor size, females and age >40 years.
- **Tumor size:** The prognosis is poor in patients with a large tumor size.
- **Site:**
  - Sacrum: Best prognosis with overall longest survival. 5 years survival rate: 60–95%, 10 years survival rate: 40–60%.
  - Mobile spine: Recurrence rate is very high as complete excision is difficult. 5 years survival rate: 55%
  - Skull base: Poor outcome. 5 years survival rate: 51%, 10 years survival rate: 35%.
- **Histological type:** Dedifferentiated chordoma has worst prognosis of all chordomas. It is rapidly fatal with systemic spread occurring in ~ 90% of cases.
- **Metastasis:** Presence of metastasis is an adverse prognostic factor. The overall rate is 5% to 43%. The most favored sites are lung, skin and bone.
- **P53 overexpression:** It is an independent factor for a higher proliferation index. Five-year survival rate is 38.9% with p53 overexpression. However, it is 79.4% in those without overexpression.

BRACHYURY (T Box Gene Family)

Function

- Promotion of cell movement and adhesion.
- Activator of transcription.

- Gene duplication is seen in familial chordoma.
- Amplification of brachyury is observed in sporadic chordoma (7%).
- No mutations have been identified yet.
- Silencing brachyury gene in experimental studies has been shown to induce growth arrest in chordoma cell lines.
- Brachyury-specific T cells can lyse human cancer cells expressing brachyury in an MHC restricted manner.
- Heat-killed, recombinant yeast-based vaccine (GI-6301) has been engineered to express brachyury.
- It is under phase 1 clinical trials.

Chromosomal Instability

LOSSES	*GAINS*
1p (21%)	7q (42%)
3p (36%)	12q (36%)
4q (27%)	17q (21%)
10q (21%)	20q (27%)
13q (24%)	22q (21%)

- Presneau et al. identified the activation of the AKT/mTOR pathway in the majority of chordomas.
- It suggests a potential therapeutic role for combined AKT and mTOR inhibitors, such as rapamycin or its analogs.
- A phase II trial by the French Sarcoma Group studying sorafenib in locally advanced and metastatic chordoma has also shown promising results.

POINTS TO REMEMBER

- Notochordal-derived lesions encompass a spectrum of benign-appearing lesions and developmental remnants.
- These include notochordal rest or notochordal remnant, along with ecchordosis physaliphora spheno-occipitalis and ecchordosis physaliphora vertebralis, to malignant neoplasia.
- Chordomas are malignant notochordal tumors with locally aggressive behavior and potential for metastasis.
- Within the current WHO classification, BNCT and chordoma comprise the entire spectrum of notochordal-derived tumors.
- However, recently recognized "atypical" NCT communicates the presence of atypical radiologic and/or histologic features.
- It should prompt careful clinical follow-up with frequent serial imaging.

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Papillary Lesions of Breast

54

Jyoti Singh

INTRODUCTION

- Papillary breast lesions are clinically, histologically, and biologically heterogeneous group of breast diseases.
- Their main common histological feature is the presence of papillae mostly with arborising fibrovascular stroma.
- They pose a diagnostic challenge especially on core biopsies.

WHO 2019 CLASSIFICATION

- Intraductal papilloma (IDP)
- Papillary DCIS
- Encapsulated papillary carcinoma
- Solid papillary carcinoma
  - In situ
  - Invasive
- Invasive papillary carcinoma.

Papillae

They are characterized by finger-like projections composed of central fibrovascular cores covered by epithelium.

PAPILLARY LESIONS

- Proliferative lesions composed of papillae.
- The spectrum of these lesions includes benign, atypical and malignant lesions (Table 54.1).
- Rarely, invasive carcinoma has papillary architecture.
- Exclusively intraductal lesions are as follows:
  - IDP
  - IDP with ADH/DCIS
  - Intraductal papillary carcinoma.
- Definite classification of encapsulated papillary carcinoma and solid papillary carcinoma is controversial.
- However, they are generally regarded as variants of **intraductal papillary carcinoma** for diagnosis and management purposes.

Table 54.1: Spectrum of papillary lesions of breast		
Hyperplasia	***Benign, presumed neoplastic***	***Neoplastic***
Papillary hyperplasia/ papillary hyperplasia of usual type	Intraductal papilloma	Papillary DCIS
	Florid papillomatosis of nipple	Solid papillary carcinoma
	Sclerosing papilloma	Encapsulated papillary carcinoma
	Above lesions with superimposed low-grade neoplasia "atypia"	Invasive papillary carcinoma

INTRADUCTAL PAPILLOMA

- Discrete benign tumors of the epithelium of mammary ducts.
- There are two types:
  - Solitary (central)
  - Multiple (periphery).

Clinical Features

Central Papilloma

- Solitary, subareolar region.
- Lactiferous ducts.
- 6th and 7th decades.
- Discharge—bloody or non-bloody in 72% of cases.
- Subareolar lump.

Peripheral Lesions

- Multiple papillomas
- They may present as a palpable lesion
- Younger age group 40–50 years.

Imaging

- Mammography/USG: Circumscribed mass in a solitary dilated retroareolar duct.
- Calcifications are rare (both large and small papillary lesions).
- USG is more sensitive than mammography.
- Galactography: Smooth filling defect or complete obstruction to retrograde flow of contrast material.
- MRI: Well-defined enhancing lesion within a fluid-filled duct.
- Radiologic features are not sufficiently sensitive or specific to differentiate benign from malignant papillary lesions.
- Combination of USG + mammography has a sensitivity of 61%, specificity of 33%, positive predictive value (PPV) of 85% and negative predictive value (NPV) 13% (Lam et al).

Gross

- Dilated duct (cyst)—with the presence of a mural nodule
- Cystic multinodular papilloma.

Microscopy

- Arborescent proliferation with epithelial and myoepithelial cells overlying fibrovascular stalks protruding into ductal lumens (finger-like projections)
- Inner myoepithelial (ME) and outer epithelial layer.

FLORID PAPILLOMATOSIS OF NIPPLE

- In and around the collecting duct.
- Clinically presents as a localized and nodular lesion.
- Sometimes in continuity with squamous epithelium as erosive adenomatosis or nipple adenoma.
- Nipple is dicharge.

Microscopy

Small/medium-sized duct with variable cystic dilation and papillary hyperplasia.

Secondary Changes

- Sclerosis
- Infarction
- Apocrine metaplasia
- Squamous metaplasia
- Mucinous
- Clear cell
- Sebaceous metaplasia.

Sclerosis

- Sclerosis in the papillary processes is diagnosed as a sclerosing papilloma.
- Amount of sclerosis is highly variable.
- It may be focal to diffuse.
- Clinically presents as a palpable lump fixed to the skin.
- Radiographically a stellate, irregular speculated abnormality is observed.
- Distorted tubules entrapped in the sclerotic areas may create a pseudo-invasive pattern.
- Persistent ME cells around these entrapped ducts confirm their benign nature.

Squamous Metaplasia

- More likely when there is infarction.
- Probably reactive or reparative process.
- Entrapped metaplastic epithelium in the stromal reaction may simulate metaplastic or squamous carcinoma.
- In some instances the distinction between these processes is very difficult.

Cytology of Papilloma

- Cellular smears.
- Complex folded, branching epithelial sheets and finger-like projections.
- True papillary fragment.

- Bare bipolar nuclei.
- Accompanied by variable amounts of apocrine cells, inflammatory cells and histiocytes.
- Apocrine cells, foamy histiocytes and bipolar nuclei (myoepithelial cells) are characteristic of a papilloma.

Diagnostic Challenge

Cytologic detection of malignancy in a papilloma:

- Often especially difficult
- Inadequate sampling from areas of atypia
- Some papillary carcinomas—cytologically benign appearance.

Treatment and Prognosis

- Excision is indicated when a core biopsy of a papillary tumor contains atypical ductal or lobular cells.
- Patients (at risk) in whom core biopsy shows a benign papilloma have a significant likelihood of harboring an atypical epithelial proliferation in an excision specimen.
- Literature suggests two features that may identify patients at elevated risk for atypia:
  - Age of patient
  - Size of the lesion.

INTRADUCTAL PAPILLOMA WITH PAPILLARY HYPERPLASIA (PAPILLOMATOSIS)

- Intraductal hyperplasia without cytologic atypia is frequently found focally or diffusely.
- When prominent, the proliferating epithelial cells often obscure the papillary nature as in **solid intraductal papilloma.**
- Secondary lumens are formed within the hyperplastic epithelium.
- These are irregular microlumens.
- At higher magnification, however, delicate fibrovascular cores with intervening ME cells are often apparent.
- In some planes of the section, the fibrovascular stalk may not be apparent at all.
- However, the following features are clues indicating a benign process:
  - Streaming or whorling of the proliferating cells
  - Irregularly distributed, often overlapping, bland nuclei
  - Inconspicuous cytoplasmic margins
  - Slit-like secondary lumens.

PAPILLOMA WITH ADH/PAPILLOMA WITH DCIS

- Papillomas can display focal proliferations of a mildly atypical, monotonous cell population.
- It is called ADH <3 mm, <30% of the duct is involved.
- DCIS >3 mm, >30% of duct involved.
- When completely excised, these 2 groups do not seem to differ in clinical behavior.

Immunohistochemistry (IHC)

- HMW cytokeratins.
- ME cell markers
- Basement membrane markers.

ME Cell Markers

- p63
- Calponin
- CD10
- Actin
- Smooth muscle myosin heavy chain (SMMHC)
- CK5/6
- All except p63 are cytoplasmic markers
- Varying reactivity with stromal cells are observed
- p63 localizes in myoepithelial cell nuclei
- Cross-reactivity in stromal cells is not observed
- Therefore, it is preferable to include a stain for p63 among those chosen to detect myoepithelial cells.
- **Calponin:** 93% sensitivity
- Due to cross-reactivity with myofibroblasts—79% specificity.
- **SMA:** 88% sensitivity
- High-cross reactivity with myofibroblasts—65% specificity
- **SMMHC:** High sensitivity, but some cross-reactivity with myofibroblasts and vascular smooth muscle.
- **HMWCK:** Ductal epithelial lining and squamous epithelium.
- **Normal breast:** CK5/6 and 34βE12 stain the ME cells and mammary duct epithelial cells.
- **Diminished to absent:** 80% to 100% of low-grade to high-grade DIN (ADH/DCIS) lesion.

Treatment and Prognosis

- Standardized incidence rates for breast carcinoma are higher for women with papillary lesions with atypia
  - Solitary with atypia—5.11
  - Multiple with atypia—7.01
  - Solitary without atypia—2.04
  - Multiple without atypia—3.01
- **Surgical excision** is advised for all papillary lesions of the breast irrespective of presence/absence of atypia.

INTRADUCTAL PAPILLARY CARCINOMA

- It is also known as papillary DCIS and non-invasive papillary carcinoma.
- 2% of all breast cancers.
- 5th and 6th decades.
- Solitary/central papillary carcinomas.
- Multifocal/peripheral type.
- Wider excision is required because of more extensive distribution of the process.

Clinical Features

- 50%—central part of the breast.
- Nipple discharge—22% to 34% of patients.
- Palpable lump.
- Slow growth rate—duration of symptoms for a year or more before presentation is not unusual.

Mammography

- Irregular intraductal/intracystic mass on USG
- Non-parallel orientation, an echogenic halo, posterior acoustic enhancement, microcalcifications.

Gross

- Cystic and solid components.
- Average 2–3 cm.
- Well-circumscribed, may even appear encapsulated.
- Soft to moderately firm, depending upon the extent of fibrosis.
- Tan or gray, hemorrhage common.

Microscopy

- Thin papillary processes, lined by atypical elongated epithelial cells.
- These cells are stratified, monomorphic and hyperchromatic.
- No discernable ME cells are seen.
- There is focal microcalcification
- p63—only rare ME cells were noted within the lesion.

INVASIVE CARCINOMA ASSOCIATED WITH PAPILLARY CARCINOMA

- Most papillary carcinomas are non-invasive.
- A small proportion shows stromal invasion.
- Invasive foci generally display the morphology of an IDC, lacking papillary features.
- The size of the invasive focus may range from microinvasive to grossly visible
- Solid sheets of invasive carcinoma cells are present in fibrofatty tissue outside a solid papillary carcinoma.

INTRACYSTIC (ENCAPSULATED) PAPILLARY CARCINOMA

- Solitary.
- Centrally located.
- Intracystic lesions that grossly exhibit a cystic space around the papillary proliferation.
- They appear circumscribed and encapsulated but lack demonstrable ME cells around the cystically dilated duct.
- Favorable prognosis with adequate local excision alone.
- Low incidence of lymph node metastasis (1–3%).

SOLID PAPILLARY CARCINOMA (SPC)

- This entity was proposed by **Maluf and Koerner** as a variant of intraductal papillary carcinoma.
- It shows compact cellular proliferation within multiple nodules that represent dilated ducts.
- Cells show low to intermediate-grade streaming.
- So, it resembles florid intraductal hyperplasia at low magnification.
- Homogeneity and significant mitotic activity—clues to the diagnosis of SPC.
- Neuroendocrine differentiation—at least focal granular eosinophilic cytoplasm.
- Immunoreactive with neuroendocrine markers.
- SPC has been hypothesized by some authors to be a possible precursor of colloid (mucinous) carcinoma of the breast.
- A frequent association has been observed between the two.
- But other types of invasive carcinoma can also be seen in association with SPCs.

EPITHELIAL DISPLACEMENT AFTER NEEDLE INSTRUMENTATION PROCEDURES

- Papillary lesions, because of their inherent friability, are particularly prone to epithelial displacement after needle instrumentation procedures.
- The epithelium can be dislodged and displaced:
  - Into the surrounding stroma
  - Adjacent lymphatic channels
  - Axillary lymph nodes.
- It is important to be aware of the possibility of displacement after needle aspiration or core biopsy.

Clues Indicating Epithelial Displacement

- History of a recent needling procedure.
- Presence of prior biopsy site changes.
- Degenerative changes in the ''invasive'' cell clusters.
- Reactive, altered stroma surrounding the fragments of epithelium.

POINTS TO REMEMBER

- Papillary lesions of the breast represent a heterogeneous group of neoplasms sharing many morphological similarities.
- They pose a diagnostic challenge, especially on core biopsies.
- However, these can usually be distinguished via careful histological evaluation.
- IHC is often required to support the diagnosis.
- Clinicoradiographic picture should be considered before giving the diagnosis.
- Excision is indicated in most of these lesions.

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Pathology of Renal Transplant Rejection

55

INTRODUCTION

- A major barrier to transplantation is the process of rejection in which the recipient's immune system recognizes graft as being foreign and attacks it.
- Rejection is a complex process in which both cell-mediated immunity and antibody-mediated humoral immunity play a role.
- The gold standard for diagnosis of rejection and guiding patient management is histological evaluation of a renal allograft biopsy.
- However, it is important to remember that graft rejection is not the only cause for renal allograft dysfunction.
- A comprehensive list of causes of renal allograft dysfunction is shown in Table 55.1.

Table 55.1: Causes of allograft dysfunction

Acute (0–6 months post-transplant, particularly 0–3 months)
Acute cellular rejection
Acute humoral rejection
Acute ischemic injury
Acute cyclosporine toxicity
Acute pyelonephritis
Chronic (>6 months after transplant)
Chronic humoral rejection (chronic cellular rejection is currently of uncertain clinical significance)
Chronic calcineurin inhibitor toxicity
Hypertension
Chronic obstruction/reflux
Chronic pyelonephritis
Polyomavirus nephropathy
Glomerular disease: – Recurrent – De nove
Graft senescence, including: – Donor-derived disease – Progression of early injury
Post-transplant lymphoproliferative disorder
Interstitial fibrosis/tubular atrophy, not otherwise specified

SPECIMEN ADEQUACY

- The biopsy is considered to be adequate if it shows an extent of tubulointerstitium containing 10 glomeruli and 2 arteries.
- Presence of 7 glomeruli and 1 artery is considered as minimally acceptable.
- The sensitivity for detection of rejection varies with the amount and type of tissue as follows:
  - 90% with one core of cortical tissue
  - 99% with two cores of cortical tissue
  - between 75% and 80% if only the medulla is present.

PROCESSING OF ALLOGRAFT BIOPSY

- The tissue is divided into three parts:
  - Light microscopy
  - Immunofluorescence microscopy (IFM)
  - Electron microscopy (EM)
- Two (2) separate cores must be obtained.
- IFM is performed to test for C4d only; full panel is performed only when glomerular disease is suspected.
- Testing for C4d is required to adequately diagnose acute humoral rejection and routine staining is essential.
- At least seven (7) slides must be examined with multiple sections:
  - Three (3) slides stained with H&E
  - Three (3) with periodic acid–Schiff stain (PAS) (with or without methenamine silver)
  - One (1) with trichrome stain.
- Sections should be cut at 2–3 μm for better appreciation of glomerular morphology.

CLINICAL HISTORY

Before the interpretation of biopsy following points in clinical history merit consideration:

- Rapidity of rise in creatinine
- Degree of sensitization
- Anti-HLA antibody status
- Previous infection
- Allograft age
- Urine findings such as decoy cells, bacteria and proteinuria.

Hyperacute Rejection

- It occurs due to preformed antidonor antibodies present in the circulation of the recipient.
- The causes of the presence of preformed antidonor antibodies are as follows:
  - A recipient who has previously rejected a kidney transplant
  - Multiparous women who develop anti-HLA antibodies against paternal antigens shed from fetus
  - Patients with a history of prior blood transfusions.
- However, with the current practice of cross-matching, hyperacute rejection is no longer a significant clinical problem.

- It occurs within minutes or hours after revascularization, immediate result being abrupt cessation of urine flow.
- Diagnosis may be obvious as mottling, cyanosis and diminished turgor in the graft.

Gross

The kidney is markedly swollen up. Cut surface is grossly hemorrhagic and glistening with edema fluid.

Microscopy

- Fibrin thrombi are present in all renal vessels including glomerular capillaries and peritubular venules.
- Thrombosis is associated with infarction and tubular necrosis.
- Glomeruli are congested and show loss of nuclei.
- There is the presence of variable infiltrate of leukocytes within glomeruli, peritubular capillaries and interstitium.

Immunofluorescence

Linear staining for IgM or IgG and C3 along glomerular and peritubular capillaries. C4d is seen along the peritubular capillaries.

Immunohistochemistry (IHC)

IHC for C4d shows widespread circumferential deposition along the peritubular capillaries.

Electron microscopy

Platelets, fibrin, sludged red blood cells and evidence of necrosis of endothelial cells of glomerular capillaries and other vessels. Degranulated neutrophils are often seen.

DIFFERENTIAL DIAGNOSIS

- The differential diagnosis of hyperacute rejection includes perfusion injury and major vascular thrombosis.
- Perfusion injury shows prominent loss of endothelium and rare thrombi but generally there is no significant hemorrhage and necrosis.
- Increased neutrophils in PTC are associated with hyperacute rejection, but not ischemic injury.
- Renal vein thrombosis shows marked congestion and relatively little neutrophil response and is not a feature of hyperacute rejection.

BANFF CLASSIFICATION

- It was developed by a group of pathologists, nephrologists and transplant surgeons at a meeting in Banff, Canada in August 1991 to codify renal allograft rejection.
- It was first published in 1993.
- Following this it continued to develop through meetings every 2 years and has become the worldwide standard for interpretation of transplant biopsies.

- Before going through the detailed classification, it is important to understand the various terms used in Banff scoring and how they are scored.
- **Glomerulitis (g):** It is an accumulation of inflammatory cells in glomeruli.
- It is scored as follows:

Score	Criteria
g0	Normal
g1	Glomerulitis in <25% glomeruli
g2	Glomerulitis in 25–75% glomeruli
g3	Glomerulitis in >75% glomeruli

- **Peritubular capillaritis (ptc):** It is the accumulation of inflammatory cells in the lumen of peritubular capillaries.
- It is scored as follows:

Score	Criteria
ptc0	At least one leukocyte in <10% of cortical PTCs and/or maximum number of leukocytes <3
ptc1	At least one leukocyte cell in ≥10% of cortical PTCs with three or four leukocytes in most severely involved PTC
ptc2	At least one leukocyte in ≥10% of cortical PTCs with five to 10 leukocytes in most severely involved PTC
ptc3	At least one leukocyte in ≥10% of cortical PTCs with >10 leukocytes in most severely involved PTC

- **Tubulitis (t):** It is the infiltration of tubular epithelium by lymphocytes.
- It is scored as follows:

Score	Criteria
t0	No mononuclear leukocytes in tubules
t1	Foci with one to four leukocytes per tubular cross-section (or 10 tubular cells)
t2	Foci with five to 10 leukocytes per tubular cross-section (or 10 tubular cells)
t3	Foci with >10 leukocytes per tubular cross-section or the presence of two or more areas of tubular basement membrane destruction accompanied by i2/i3 inflammation and t2 elsewhere

- **Intimal arteritis (v):** Intimal thickening with inflammation of arterial subendothelial space.
- It is scored as follows:

Score	Criteria
v0	No arteritis
v1	Mild to moderate intimal arteritis in at least one arterial cross-section
v2	Severe intimal arteritis with at least 25% luminal area lost in at least one arterial cross-section
v3	Transmural arteritis and/or arterial fibrinoid change and medial smooth muscle necrosis with lymphocytic infiltrate in vessel

- **C4d immunofluorescence (C4d):** It is seen in the form of fluorescence in the peritubular capillaries.
- It is scored as follows:

Score	Criteria
C4d0	No staining of PTCs (0%)
C4d1	Minimal C4d staining (>0 but <10% of PTCs)
C4d2	Focal C4d staining (10–50% of PTCs)
C4d3	Diffuse C4d staining (>50% of PTCs)

- **Mesangial matrix expansion (mm)** is scored as follows:

Score	Criteria
mm0	No more than mild mesangial matrix increase in any glomerulus
mm1	At least moderate mesangial matrix increase in up to 25% of nonsclerotic glomeruli
mm2	At least moderate mesangial matrix increase in 26–50% of nonsclerotic glomeruli
mm3	At least moderate mesangial matrix increase in >50% of nonsclerotic glomeruli

- **Arteriolar hyalinosis (ah):** It is PAS-positive hyaline thickening in the arterioles.
- It is scored as follows:

Score	Criteria
ah0	No PAS-positive hyaline arteriolar thickening
ah1	Mild to moderate PAS-positive hyaline thickening in at least one arteriole
ah2	Moderate to severe PAS-positive hyaline thickening in more than one arteriole
ah3	Severe PAS-positive hyaline thickening in many arterioles

- **Vascular fibrous intimal thickening (cv)** is scored as follows:

Score	Criteria
cv0	No chronic vascular changes
cv1	Vascular narrowing of up to 25% luminal area by fibrointimal thickening
cv2	Vascular narrowing of 26–50% luminal area by fibrointimal thickening
cv3	Vascular narrowing of >50% luminal area by fibrointimal thickening

- **Interstitial fibrosis (ci)** is scored as follows:

Score	Criteria
ci0	Interstitial fibrosis in up to 5% of cortical area
ci1	Interstitial fibrosis in 6–25% of cortical area (mild interstitial fibrosis)
ci2	Interstitial fibrosis in 26–50% of cortical area (moderate interstitial fibrosis)
ci3	Interstitial fibrosis in >50% of cortical area (severe interstitial fibrosis)

- **Tubular atrophy (ct):** It is the presence of renal tubules with thick redundant basement membranes or a reduction of tubular diameter by >50% as compared to normal tubules.
- It is scored as follows:

Score	*Criteria*
ct0	No tubular atrophy
ct1	Tubular atrophy involving up to 25% of the area of cortical tubules (mild tubular atrophy)
ct2	Tubular atrophy involving 26–50% of the area of cortical tubules (moderate tubular atrophy)
ct3	Tubular atrophy involving >50% of the area of cortical tubules (severe tubular atrophy)

- **Double contouring (cg)** of glomerular basement membrane (GBM).
- It is scored as follows:

Score	*Criteria*
cg0	No GBM double contours by light microscopy or EM
cg1a	No GBM double contours by light microscopy but GBM double contours (incomplete or circumferential) in at least three glomerular capillaries by EM, with associated endothelial swelling and/or subendothelial electron-lucent widening
cg1b	Double contours of the GBM in 1–25% of capillary loops in the most affected nonsclerotic glomerulus by light microscopy; EM confirmation is recommended if EM is available
cg2	Double contours affecting 26–50% of peripheral capillary loops in the most affected glomerulus
cg3	Double contours affecting >50% of peripheral capillary loops in the most affected glomerulus

- **Interstitial inflammation (i)** is scored as follows:

Score	*Criteria*
i0	No inflammation or in <10% of unscarred cortical parenchyma
i1	Inflammation in 10–25% of unscarred cortical parenchyma
i2	Inflammation in 26–50% of unscarred cortical parenchyma
i3	Inflammation in >50% of unscarred cortical parenchyma

- **Interstitial fibrosis and tubular atrophy (i-IFTA)** are scored as follows:

Score	*Criteria*
i-IFTA0	No inflammation or <10% of scarred cortical parenchyma
i-IFTA1	Inflammation in 10–25% of scarred cortical parenchyma
i-IFTA2	Inflammation in 26–50% of scarred cortical parenchyma
i-IFTA3	Inflammation in >50% of scarred cortical parenchyma

- The most recent updated 2015 Banff classification is summarized in Table 55.2 and is discussed subsequently.

Table 55.2: Updated 2015 Banff classification categories

Category 1	Normal biopsy or nonspecific changes
Category 2	Antibody-mediated changes Acute/active antibody-mediated rejection (ABMR) Chronic active ABMR
Category 3	Borderline changes
Category 4	T cell mediated rejection (TCMR)
Category 5	Interstitial fibrosis and tubular atrophy
Category 6	Other changes not considered to be caused by acute or chronic rejection

Category 2: Antibody-Mediated Changes

Acute/Active Antibody-Mediated Rejection (ABMR)

- All three features must be present for diagnosis.
- Biopsies showing histological features plus evidence of current/recent antibody interaction with vascular endothelium or DSA, but not both, may be designated as suspicious for acute/active ABMR.
- Lesions may be clinically acute or smoldering or may be subclinical.
- It should be noted if the lesion is C4d-positive or C4d-negative.

Diagnostic Criteria

- Histologic evidence of acute tissue injury, including one or more of the following:
  - Microvascular inflammation (g >0 in the absence of recurrent or de novo glomerulonephritis, and/or ptc >0)
  - Intimal or transmural arteritis (v >0)
  - Acute thrombotic microangiopathy in the absence of any other cause
  - Acute tubular injury in the absence of any other apparent cause
- Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
  - Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections or C4d >0 by IHC on paraffin sections)
  - At least moderate microvascular inflammation ([g + ptc] ≥2), although in the presence of acute TCMR, borderline infiltrate, or infection; ptc ≥2 alone is not sufficient, and g must be ≥1
  - Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
- Serologic evidence of DSAs (HLA or other antigens)
  - Biopsies suspicious for ABMR on the basis of meeting criteria 1 and 2 should prompt expedited DSA testing

Chronic Active Antibody-Mediated Rejection (ABMR)

- All three features must be present for diagnosis.
- Biopsies showing histological features plus evidence of current/recent antibody interaction with vascular endothelium or DSA, but not both, may be designated as suspicious.
- The status of C4d whether C4d-positive or C4d-negative should be noted.

Diagnostic Criteria

- Histologic evidence of chronic tissue injury, including one or more of the following:
  - Transplant glomerulopathy (cg >0), if no evidence of chronic thrombotic microangiopathy; includes changes evident by EM only (cg1a)
  - Severe peritubular capillary basement membrane multilayering (requires EM)
  - Arterial intimal fibrosis of new onset, excluding other causes; leukocytes within the sclerotic intima favor chronic ABMR if there is no prior history of biopsy-proven TCMR with arterial involvement but are not required.
- Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
  - Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d >0 by IHC on paraffin sections)
  - At least moderate microvascular inflammation ([g + ptc] ≥2), although in the presence of acute TCMR, borderline infiltrate, or infection, ptc ≥2 alone is not sufficient and g must be ≥1
  - Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
- Serologic evidence of DSAs (HLA or other antigens):
  - Biopsies suspicious for ABMR on the basis of meeting criteria 1 and 2 should prompt expedited DSA testing

C4d Staining Without Evidence of Rejection

All three features must be present for diagnosis

- Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d >0 by IHC on paraffin sections)
- g = 0, ptc = 0, cg = 0 (by light microscopy and by EM if available), v = 0; no TMA, no peritubular capillary basement membrane multilayering, no acute tubular injury (in the absence of another apparent cause for this)
- No acute cell-mediated rejection (Banff 1997 type 1A or greater) or borderline changes.

Category 3: Borderline Changes

Suspicious for Acute TCMR

- Foci of tubulitis (t1, t2, or t3) with minor interstitial inflammation (i0 or i1) or interstitial inflammation (i2, i3) with mild (t1) tubulitis; retaining the i1 threshold for borderline from Banff 2005 is permitted although this must be made transparent in reports and publications
- No intimal arteritis (v = 0)

Category 4: TCMR

Acute TCMR

It is graded as follows:

- Grade IA. Significant interstitial inflammation (>25% of nonsclerotic cortical parenchyma, i2 or i3) and foci of moderate tubulitis (t2).
- IB. Significant interstitial inflammation (>25% of nonsclerotic cortical parenchyma, i2 or i3) and foci of severe tubulitis (t3).
- IIA. Mild to moderate intimal arteritis (v1) with or without interstitial inflammation and Tubulitis.
- IIB. Severe intimal arteritis comprising >25% of the luminal area (v2) with or without interstitial inflammation and Tubulitis.
- III. Transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3).

Chronic Active TCMR

- Chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neointima).
- It is to be noted that such lesions may represent chronic active ABMR as well as TCMR.
- The latter may also be manifest in the tubulointerstitial compartment.

Category 5: Interstitial Fibrosis and Tubular Atrophy

It is further graded as follows:

- **Grade I:** Mild interstitial fibrosis and tubular atrophy (≤25% of cortical area).
- **Grade II:** Moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area).
- **Grade III:** Severe interstitial fibrosis and tubular atrophy (>50% of cortical area).

Category 6: Other Changes not Considered to be Caused by Acute or Chronic Rejection

These include the following:

- BK virus nephropathy
- Posttransplant lymphoproliferative disorders
- Calcineurin inhibitor nephrotoxicity
- Acute tubular injury
- Recurrent disease
- De novo glomerulopathy (other than transplant glomerulopathy)
- Pyelonephritis
- Drug-induced interstitial nephritis.

BK Virus Nephropathy

- It is characterized by the presence of characteristic morphological changes especially in the nuclei.
- These include the following:
  - Amorphous ground-glass inclusion body
  - Central irregular inclusion body
  - Finely granular nuclear alterations
  - Vesicular nuclei with coarsely clumped viral inclusions.

- Urine shows the characteristic Decoy cells.
- These cells show glassy-appearing intranuclear viral inclusion bodies, sometimes interspersed with dense, granular material.
- They are a morphologic sign of the activation of polyomaviruses.

Adenovirus Infection

- It shows focal necrotizing tubulointerstitial nephritis.
- Occasional small granulomas may be seen.
- Ground glass type of intranuclear viral inclusion bodies in tubular epithelial cells, tubular destruction, interstitial hemorrhage, and necrosis are the other features.
- IHC may be done to detect adenovirus antigens.

Cytomegalovirus (CMV) Infection

It shows the characteristic round and discrete intranuclear inclusions surrounded by a thin white halo.

Posttransplant Lymphoproliferative Disorder (PTLD)

- PTLD often presents with a nodular, destructive-appearing mononuclear cell infiltrate.
- On higher magnification activated lymphoid cells and scattered blasts are seen.

Calcineurin Inhibitor Nephrotoxicity

- The characteristic feature is isometric vacuolization of the proximal tubular epithelial cell cytoplasm.
- In comparison, ischemia-induced tubular injury with nonisometric vacuolization of the tubular cytoplasm; this change should not be mistaken for toxic tubulopathy.
- Peripheral glomerular capillary walls show segmental duplication without conspicuous cell interposition that is only occasionally seen.
- Afferent arterioles feeding the affected glomeruli characteristically demonstrate CNI-induced toxic arteriolopathy.
- Arteriolopathy suggestive of late CNI-induced toxicity shows PAS-positive hyaline deposits deep in the medial smooth muscle layers.
- Some of the arterioles show a "string of pearl"-like arrangement of hyaline nodules along the adventitial aspect.
- Other cases show very severe transmural and circumferential hyaline accumulations.

Drug Allergy

- Definitive distinction between drug allergy and cellular rejection is not generally possible.
- If eosinophils are prominent (greater than 5%), if eosinophils invade tubules, or if inflammation is centered at the corticomedullary junction and not in the renal cortex, then the possibility of drug allergy should be considered (in the absence of transplant endarteritis and lack of PTC C4d staining).

Mixed Rejection

- Biopsies that show features of both Category 2 and Category 4 that are for both ABMR and TCMR type.
- These mixed rejection episodes are quite common.

C4d-negative ABMR

- In certain cases of ABMR C4d is found to be absent.
- However, presence of DSA and microcirculation inflammation in the absence of immune complexes in glomeruli and tubular basement membranes make ABMR the most likely diagnosis.

POINTS TO REMEMBER

- Histopathological examination of allograft biopsy is the gold standard for evaluation of graft dysfunction.
- A meticulous approach following the updated Banff 2015 classification helps in reaching an objective diagnosis.
- Application of immunofluorescence for C4d and electron microscopy plays a crucial role in many cases.
- The goal of the Banff process is the integration of advances in histologic, serologic and molecular diagnostic techniques.
- This produces a consensus-based reporting system that offers precise composite scores, accurate routine diagnostics, and applicability to next-generation clinical trials.

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Section

7

Cytopathology

Cell Block Preparation Techniques

56

Poorvi Kapoor

INTRODUCTION

- Cell blocks are microbiopsies embedded in paraffin that broaden the diagnostic value of cytology specimens and are complimentary to cytology preparations.
- It employs retrieval of small tissue fragments from FNA specimens which are further processed to form a paraffin block.
- It increases the diagnostic sensitivity and specificity (through both cellular morphology and ancillary testing).
- It requires minimal effort and is extremely cost-effective. The tissue preserved in a cell block can be readily shared for second opinions and research without fear of losing the original smear specimen.

Advantages

- Cell block preparation is simple, reproducible and readily available in routine laboratories.
- There is increased cellularity with better morphology and architecture patterns.
- This increased cellularity helps increase the diagnostic yield and accuracy.
- It allows for unlimited storage of the sample.
- Ancillary tests, like immunohistochemistry and molecular studies, can be performed on cell block preparations.

Specimens

- Almost all routine specimens in cytology can be processed to make a cell block from the sediment available.
- Fine needle aspiration cytology and biopsy specimens.
- Sputum.
- Urine.
- Effusion fluids, like pleural, peritoneal and pericardial.
- Bronchial lavages, brushings and washings.
- Pelvic and abdominal washings.

Methods

Fixed Deposit Method

- The sample/sediment is fixed in formalin and centrifuged at 2500 rpm for 10 minutes.

- The supernatant is poured off and the sediment is removed, packed in lens paper and put it in a labeled tissue cassette.
- It is further processed as a routine specimen.

Agar Method (3% Agar)

- The sample is centrifuged, excess supernatant is removed
- 3% molten agar is added to the sediment and mixed. It is then refrigerated to get a solid button
- The button is placed in 10% formalin and processed as tissue

Simplified Cell Block Technique

- Fix the sample with 50% alcohol in a conical centrifugation tube for 1 hour.
- Centrifuge for 7 minutes at 300 g and pour off supernatant.
- Re-suspend the pellet for 10 minutes in 3 mL acetone.
- Centrifuge the sample again for 10 minutes at 300 g and pour off acetone.
- Place the tubes for an hour on a warm plate, then add melted paraffin to the warm pellet.
- After solidification, tap the bottom of the tube to remove the block.
- Cut and process the conical end of the paraffin block as a routine specimen.
- This technique is easy and minimal cell loss is seen with this method.

Plasma Thrombin Clot Method

- Take out-dated plasma from the blood bank and mix well with fresh, unfixed sediment.
- Add equal drops of thrombin as plasma and mix well.
- Mixture will clot in 1–2 minutes, fold this clot in lens paper and process it as tissue.

Compact Cell Block Technique

- This method produces a cell block 10–20% the size of a conventional cell block
- Cells are packed into an area which is free of erythrocytes and extracellular protein, reducing screening time
- Take 40 mL of the sample, centrifuge and pour off the supernatant.
- Add an equal volume of cyto rich red.
- After 2 minutes, add 4 drops of plasma and 3 drops of thrombin and gently agitate.
- Once clot forms, place clot on filter paper, place in fixative and process as tissue.

SHIDHAM'S METHOD

Advantages

- It involves steps to concentrate the cells along the plane parallel to the cutting surface of the cell block.
- It includes a beacon like an AV-marker which helps visualize the area of interest.
- This beacon also serves as a locator reference point in serial sections. It helps act as a beacon to locate a particular group of cells.

Preparation of the Sample

- Transfer the residual liquid-based cytology specimen to a flat bottom glass tube (15 mm diameter × 45 mm). Place this glass tube into a larger plastic carrier tube (28 mm × 85 mm) and centrifuge.
- Remove the glass tube and pour off the supernatant. Cap the glass tube and place it back inside the carrier tube and cap the carrier tube as well.
- Centrifuge at 3000 rpm for 5 minutes.
- Remove the tubes vertically from the centrifuge and remove the glass tube with forceps without disturbing the sedimented pellet with cells.
- Pour off the supernatant carefully from the glass tube without disturbing the sediment at the bottom.

Inclusion of the Reference Coordinate AV-marker and Addition of Gel

- A dark beacon AV-marker (which is a dark-colored, sectionable material around 2 mm × 2 mm in size) is added as a signpost to the glass tube.
- Liquefy an aliquot of HistoGel (HG) by melting it in a microwave for 10 seconds and add 0.5 mL of this molten HG to the sediment.
- Mix well and re-cap quickly without allowing the HG to solidify.
- Add 2.5 mL of warm water to the carrier tube and place the glass tube inside this.
- Centrifuge and spin at 3000 rpm for 5 minutes. This is to push the AV-marker, concentrate the cells and form a layer close to the cutting surface of the final paraffin embedded cell block.
- Remove the glass tube from the centrifuge and refrigerated in a vertical position to cool and solidify the HG.

Removal of the Cell Block as a Button of Gel with Specimen for Final Processing

- The solidified HG disc with the sediment is dislodged from the glass tube by inserting the needle along the periphery of this disc and rotating it along the side of the tube and squirting 10% formalin through a 23 G needle.
- This dislodges the concentrated specimen with the HG button and dark colored beacon AV-marker.
- This button is now processed as routine tissue.

Preparation of AV-marker from Banana Peels

- Take fresh peels from a raw banana with thick skin and cut the peel longitudinally into 2 mm wide strips.
- Divide each strip into 2 mm short segments and collect them in a small container.
- Add India Ink to this till each piece is coated with the ink and allow it to stand for 10 minutes.
- Add 10% formalin and remove extra ink by washing it with water or formalin and store these peels in 10% formalin.

CYTOFOAM METHOD

- A cytofoam core needle device is used which is a 23 G needle with an adsorbent support.

- This can be inserted into the hub of a needle and the material is adsorbed onto this polyvinyl alcohol foam.
- The foam can then be removed, fixed in formalin and processed as a routine tissue.

Collodion Cell Block Technique

- Collodion is a flammable, syrupy solution of pyroxylin (i.e. nitrocellulose) in ether and alcohol.
- The collodion bag is prepared by pouring collodion into a test tube and pouring it back into the bottle containing collodion by rotating the test tube, thus ensuring that the test tube is coated with collodion.
- The test tube is drained of excess collodion by inverting it and allowing it to dry for 10–15 minutes.
- Once dry, fill the tube with distilled water and cover the tube with paraffin to seal it. This prepared collodion bag can be stored for up to 1 week.
- The formalin-fixed cellular material is transferred to this bag after discarding the distilled water and capped with the paraffin.
- It is centrifuged at 2500 rpm for 10 minutes.
- The cell block bag is removed from the tube and tied just above the pellet with a thread and the excess bag is trimmed with scissors and placed on filter paper and processed as routine tissue.

Cellient Automated Cell Block System

- The Cellient® Automated Cell Block System creates a paraffin-embedded cell blocks by using vacuum filtration to deposit a layer of cells on a filter, and infiltrate these cells with processing reagents and paraffin.
- This technique is comparable with the agar method and is found to demonstrate better morphological details.

UTILITY OF CELL BLOCKS

- Various studies have found that cell blocks have an additional diagnostic yield in malignant effusions by nearly 10–13%.
- Cell blocks aid in the diagnosis and sub-classification of lymphomas.
- This technique also helps classify lung carcinomas according to the WHO 2015 classification
- Good correlation and reliability is seen with IHC performed on cell blocks for various tumors.

POINTS TO REMEMBER

- Cell blocks act as a bridge between cytopathology and histopathology.
- Cell blocks prepared from residual tissue fluids act as an adjunct to smears for establishing a definitive diagnosis.
- The technique is simple, safe, efficacious, cost-effective and reproducible even in resource limited settings.
- With newer techniques, there is better material retrieval and ancillary techniques have improved diagnostic accuracy in the field of cytopathology.

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57 Diagnostic Role of Endometrial Cell Block Cytology

WHAT IS CELL BLOCK?

- Cell blocks are micro biopsies embedded in paraffin.
- They broaden the diagnostic value of cytology specimens and are complementary to cytology preparations.
- Retrieval of small tissue fragments from FNA specimen is performed which are subsequently processed to form a paraffin block.

NEED FOR CELL BLOCK

- Acquisition of tissue for cell block increases both diagnostic sensitivity and specificity (through both tissue architecture and ancillary testing).
- It requires minimal effort and is extremely cost-efficient.
- Moreover, tissue preserved in cell block can be readily shared for a second opinions and research without fear of losing the original diagnostic smear specimen.

Advantages

- It is simple, reproducible and readily available in routine cytology laboratories.
- There is the presence of increased cellularity.
- Better morphological details and architectural patterns can be appreciated.
- Additional yield of cells, thereby increasing diagnostic yield.
- Unlimited storage of the sample since it is paraffin-embedded.
- Application of ancillary studies (IHC and molecular testing) can be performed on the cytologic specimen.

SPECIMENS

- FNAC/FNAB
- Sputum
- Urine
- Effusion fluids
- Lavages and washings.

CELL BLOCK METHODS

1. Fixed deposit method
2. Agar method
3. Simplified cell block technique
4. Plasma thrombin method
5. Compact cell block technique

Fixed Deposit Method

1. Mix sediment in a fixative
2. Centrifuge at 2500 rpm for 10 minutes.
3. Discard supernatant.
4. Carefully remove the sediment with a spatula and wrap it in a lens paper
5. Put into a cassette and put into fixative.
6. Process it as tissue sections.

Agar Method

Steps 1–3 same as in sediment method

4. Remove sediment using a spatula and place it conical side up on a paper towel.
5. Slice the sediment into half using a scalpel.
6. Place cut side in molten agar spread on a glass slide/petri dish. Cover all areas with agar and leave it to harden.
7. Trim excess agar and place it in a cassette and process.

Simplified Cell Block Technique

1. In 50 mL conical tube add cell sample with 50% alcohol for 1 hour.
2. Centrifuge @ 300 g for 7 minutes. Pour off supernatant.
3. Resuspend in 3 mL of acetone for 10 minutes.
4. Spin @ 300 g for 10 minutes. Pour off supernatant.
5. Place tubes on a warm plate for 1 hour.
6. Add melted paraffin.
7. After paraffin has solidified, tap bottom to remove block.
8. Cut and process the conical end of the block.

Plasma Thrombin Method

1. Add a few drops of plasma with fresh unfixed sediment.
2. Add same no. of drops of Thrombin and mix.
3. Allow to stand for 1–2 minutes for solution to clot.
4. Place clot in filter paper and transfer into a cassette.
5. Process the tissue.

Compact Cell Block Technique

1. Centrifuge 40 cc of a well-mixed aliquot of sample and pour off the supernatant.
2. Mix the sediment with an equal volume of Cyto Rich Red.
3. After 2 minutes, add 4 drops of plasma and 3 drops of thrombin.
4. Gently agitate the mixture when clotting stops, place clot on a filter paper.
5. Fold the filter paper, press hard, wrap it in a filter paper and place it in fixative.

SAMPLING DEVICES

- Dilation and curettage has been used clinically for many years.
- Originally it was intended to serve as a screening tool for endometrial carcinoma, but researchers realized that specimen adequacy and diagnostic accuracy is quite unsatisfactory.
- The other drawbacks of this technique include the following:
  - D&C fails to detect focal lesions of the endometrium.
  - Pain and severe discomfort during the procedure.
  - Persistent bleeding for several days after the procedure.

List of Sampling Devices

- Tao brush
- Li brush
- Endocyte
- Endoflower
- SAP 1
- Galvees negative pressure jet wash
- Mi-Mark and Isaacs instruments
- Mendhosa cannula
- Infant feeding tube.

ENDOMETRIAL CYTOLOGY

- Endometrial cytology belongs to the most difficult areas of study of cell morphology.
- There are two main reasons for it:
  - The difficulties with obtaining a representative sample of the endometrium
  - Inconspicuous differences between normal and abnormal endometrial cells because of the small size of the cells.
- Compared to cervical cytology, endometrial cytology is considered diagnostically unreliable.
- The changes in the cellular features and the presence of cell clumps during normal menstrual cycle further complicate matters.
- In developed countries, endometrial cancer morbidity has exceeded that of cervical cancer to become the most common invasive malignancy of the female genital tract.
- Early screening methods in cervical cancer have made major progress because of the improvement of cytologic preparation methods and unified evaluation system in the 1950s.
- Drawing from the successful experience of cervical carcinoma screening, there is a need for an effective approach to screening endometrial carcinoma and its precursor lesions to reduce new cases and deaths.

Morphology

Proliferative Phase Endometrium

1. **Glandular cells** are seen in cohesive monolayered sheets.
2. They appear as straight or twisted tubular structures resembling glove-finger pattern.

3. They are usually open at both ends (open type). Rarely, closed at one end (closed type) and have a cup-like/half moon/spherical shape.
4. The nuclei are uniform in size and shape with granular chromatin and distinct micro-nucleoli.
5. **Stromal cells** are seen in the form of loose aggregates.
6. They have oval nuclei and poorly defined cytoplasm.
7. In the late proliferative phase, sheets of endometrial cells are highly cellular with nuclear crowding, denser nuclear chromatin and frequent mitotic figures.

Secretory Phase Endometrium

1. Glandular cells become larger with a well-defined clear cytoplasm, vacuolizations and finely granular chromatin.
2. Stromal cells are spindle-shaped and occur in loose sheets.

Menstrual Phase Endometrium

1. Bloody smear with many ball-like tissue fragments (menstrual cell balls) consisting of degenerated glandular cells surrounded by pre-decidual cells are seen.
2. Stromal cells have ovoid, small, pyknotic nuclei and may appear as aggregates.
3. Neutrophils and nuclear debris are present in the background.

Atrophic Endometrium

1. The sheets of endometrial cells show spacing between nuclei, characteristic of atrophy.
2. Mono- and multinucleated macrophages are occasionally observed in such smears.

Simple Hyperplasia

1. There is little increase in cellularity, with cells arranged in small and large groups/sheets with cell cohesion.
2. Cystic dilatation in tissue fragments.
3. Nuclei show mild to moderate anisonucleosis, fine granulation, even chromatin, and scant cytoplasm.

Complex Hyperplasia

1. Increased cellularity, piling up of cells and increase in the size of cells are seen.
2. Enlargement of nuclei, fine granular chromatin, and macronucleation with mild variation of nuclear size are observed.

Atypical Endometrial Hyperplasia

1. Hypercellularity of smears, 3D structures, glandular and papillary formation of cells.
2. Increased N:C ratio, granular chromatin, presence of nucleoli, nuclear crowding and disturbed nuclear polarity are seen.

Endometrial Carcinoma

1. Disorganization of cells in sheets and 3D clusters with extreme variation in shape and size of cells.

2. Disturbed nuclear polarity along with hyperchromasia, increased nuclear chromatin granularity and increased number of macronucleoli.
3. There is a loss of cohesiveness and abnormal mitotic figures.

- Currently, for endometrial lesions, there are several quantitative and qualitative cytological methods that are based on the cell-collecting techniques of individual laboratories or hospitals.
- However, there is no inclusive diagnostic reporting system for endometrial cytology that has clear definitions of specimen quality.
- Some authors have proposed diagnostic criteria.
- These consist of two main elements:
  1. Criteria that reflect the cytoarchitecture
  2. Conventional criteria (background, atypia of cells, or cell clumps).
- Cell clumps were classified into two categories:
  - *Normal cell clumps:* Cell clumps with a tube or sheet-shaped pattern.
  - *Abnormal cell clumps:* Cell clumps with dilated or branched patterns, papillotubular patterns and irregular protrusions.

ROLE OF CELL BLOCK (CB)

- Both the morphology of endometrial cells and glandular architecture are critical for the diagnosis of endometrial lesions.
- CB maintains cell morphology and tissue architecture.
- Thus, it is a useful complement to cytological smears for definitive diagnosis.
- CB slides can preserve both cellular (nuclear and cytoplasm) characteristics and architectural patterns of endometrial glands and stroma, thereby increasing the diagnosis accuracy.

Advantages of Cell Block

- Cell block shows a better diagnostic accuracy as compared to LBC in almost all the endometrial lesions studied thus far.
- Diagnosis of Endometrial carcinoma has shown 100% accuracy by CB preparation.
- CB preparations allow the identification of **hobnail** cells easily, thus allowing a specific diagnosis of clear cell carcinoma.
- The presence of **squamous and adenomatous** components in a case of adenosquamous carcinoma is more apparent in CB preparations.
- Ancillary techniques like IHC can be easily put in case of confusion on the origin of the tumor which is usually endometrial (ER, vimentin and CK7) vs endocervical (p16, CEA and HPV testing).
- Multiple sections can be taken whenever needed.
- CB ensures maximum utilization of the available diagnostic material.
- The aspirated material can be easily stored as cell blocks.
- CB can also be easily combined with the LBC technique.
- Combined use further improves the diagnostic accuracy in comparison with either technique performed alone.
- An adequate gynecologic sample for CB preparation frequently can be obtained from the original vial after preparation of the cytologic LBC slide.

Disadvantages of Cell Block

- Degeneration of the cells due to delay in fixation of the aspirate.
- Cellular details are not as clear as in a biopsy specimen.
- Since tissue processing is involved it leads to a further delay in the diagnosis.

POINTS TO REMEMBER

- Endometrial cytology still remains a non-standardized method of diagnosis due to the lack of properly laid down diagnostic criterion.
- However, few countries like Japan have accepted it as a method of screening in endometrial cancers.
- Till now only a few studies have been conducted studying the role of cell block as an adjunct with cytological examination.
- This is due to the better preservation of cytoglandular architecture and the ability to use ancillary techniques.
- Since cytology allows better preservation of cellular details and cell block allows better viewing of the tissue architecture, promising results can be obtained with combination of the two techniques.
- However, since it involves tissue processing and further ancillary testing the diagnosis will get delayed.

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58 Comparative Analysis of Molecular Techniques for Human Papillomavirus (HPV) in Cytology

Saba Naaz

INTRODUCTION

- HPV is causally involved in the development of almost all cases of cervical cancer.
- The role of human papillomaviruses (HPVs) in the aetiology of invasive cervical carcinoma has been well established.
- At least 13 genotypes of the alpha genus (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) have been found to be associated with the risk of developing cervical cancer and defined as "carcinogenic" viral types.
- HPV16 is the most prevalent genotype in both squamous cell carcinoma (53.9%) and adenocarcinoma (36.3%) across the world.
- HPV18 is the second most common genotype.
- It has been found in a higher proportion of adenocarcinoma (36.8%) compared to squamous cell carcinoma (13.2%).

EPIDEMIOLOGY OF CERVICAL CANCER

- Globally, 6,04,000 women are infected with genital human papillomavirus.
- In India ~ 1,22,844 new cases of cervical cancers are diagnosed every year most of which are HPV-related.
- About 3,42,000 deaths occur worldwide and 72000 deaths occur in India every year due to HPV-related infections.

STRUCTURE OF HUMAN PAPILLOMAVIRUS

- It is a small, non-enveloped, dsDNA virus.
- It belongs to the papillomaviridae family.
- It is called papillomavirus as certain types may cause warty growths and papillomas.
- 55 nm icosahedral capsid composed of 72 capsomers (60 hexameric + 12 pentameric).
- Each capsomere is composed of 2 capsid proteins, 1 major (encoded by the L1 gene) and 1 minor.
- There are two types of HPV: Low-risk and high-risk.
- The differences between the two are shown in Table 58.1.

Table 58.1: Differences between low-risk and high-risk HPV

High-risk HPV	*Low-risk HPV*
Oncogenic HPV types integrate into DNA of the cell and modify its behavior and lead to cancer.	These types do not integrate into DNA, thus cause only skin and genital warts (condyloma)
Examples: 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82	1, 2, 3, 4, 6, 7, 8, 11, 32, 42, 44, 53, 63

PATHOGENESIS

- The expression of E6 and E7 genes, causes deregulation of cell cycle control through the spinous and granular epithelial layers, inducing differentiating cells to enter into S phase and allowing amplification of the viral genome (Fig. 58.1).
- The late proteins L1 and L2 are actively expressed in the cornified layers where virions are shed after the encapsidation of newly synthesized viral genome.
- The productive HPV infection could be clinically associated with changes in the epithelial morphology leading to development of benign hyperproliferative flat warts, condylomata, or papillomas.
- In most women immune response to HPV infection develops after a period of months or years and results in adequate viral clearance.
- The pattern of viral gene expression of high-risk HPVs in low-grade squamous intraepithelial lesions (LSIL) is very similar to that seen in productive warts.
- Conversely, high-grade intraepithelial lesions (HSIL) and invasive cancer represent abortive infections in which early genes E6 and E7, but not late genes L1 and L2, are expressed in all mucosal epithelial layers and the normal life cycle of the virus cannot be completed.
- E7 mainly appears to be associated with malignant progression of infected cells and with multiple alterations in viral and cellular pathway.

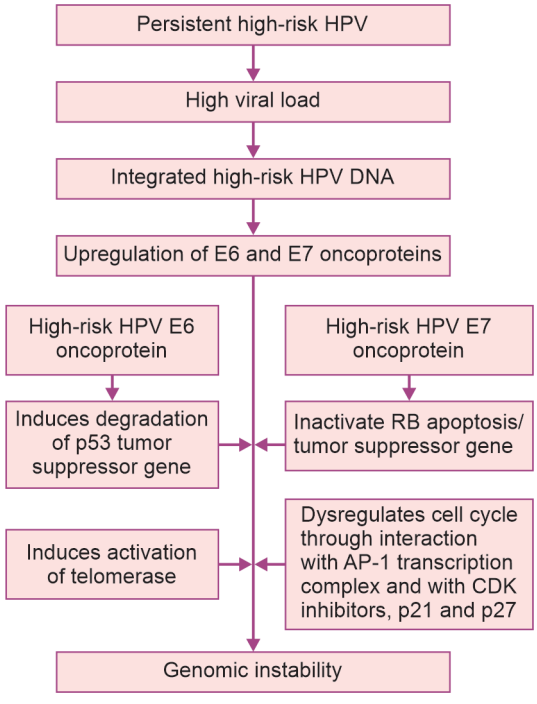
Persistent high-risk HPV
High viral load
Integrated high-risk HPV DNA
Upregulation of E6 and E7 oncoproteins
High-risk HPV E6 oncoprotein
High-risk HPV E7 oncoprotein
Induces degradation of p53 tumor suppressor gene
Inactivate RB apoptosis/ tumor suppressor gene
Induces activation of telomerase
Dysregulates cell cycle through interaction with AP-1 transcription complex and with CDK inhibitors, p21 and p27
Genomic instability

Fig. 58.1: Pathogenesis of cervical cancer by HPV virus

GUIDELINES FOR CERVICAL CANCER SCREENING

- According to the American Cancer Society 2020 cervical cancer screening starts after the age of 21 years.
- Either cytology alone or in combination with HPV testing (co-testing) is used for cervical screening (Table 58.2).
- Cervical cancer screening helped in decreasing the mortality rate due to early detection of cancer.

Table 58.2: Cervical cancer screening guidelines (ACS 2020 and ACOG 2018 guidelines)

Age	*ACS (2020)*	*ACOG (2018)*
<21 years	Not recommended	Not recommended
21–29 years	Start at 25 years Primary HPV testing alone every 5 years (preferred) or Co-testing every 5 years or Cytology alone every 3 years	Start at 25 years Cytology alone every 3 years
30–65 years	Primary HPV testing alone every 5 years (preferred) or Co-testing every 5 years or Cytology alone every 3 years	Primary HPV testing alone every 5 years (alternative) or Co-testing every 5 years (preferred) or Cytology alone every 3 years
>65 years	Not recommended	Not recommended
Hysterectomy (with no history of cervical cancer or high-grade precancerous lesion)	Not recommended	Not recommended

PRIMARY HPV SCREENING ALGORITHM

The primary screening algorithm followed in India is shown in Fig. 58.2.

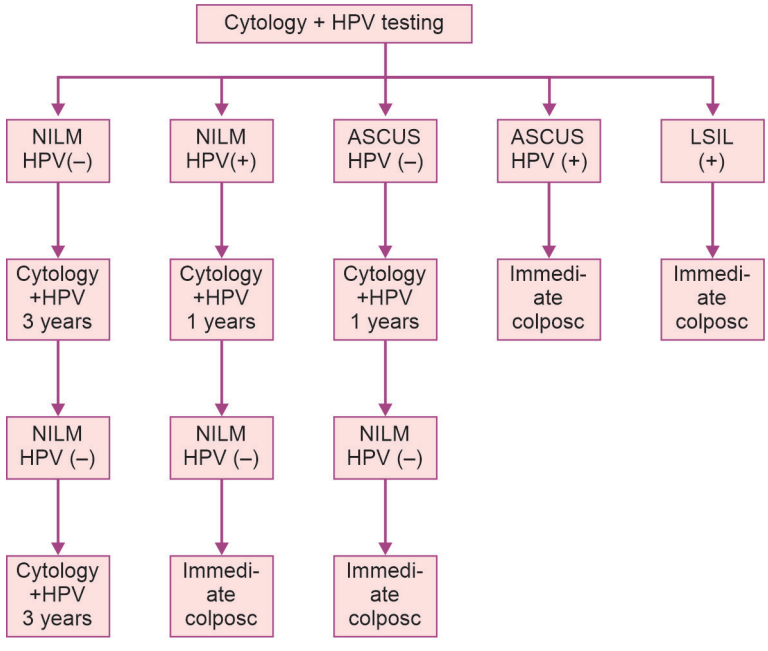

Fig. 58.2: Primary screening algorithm followed in India

Negative for Intraepithelial Lesion or Malignancy (NILM); Atypical Squamous Cells of Undetermined Significance (ASCUS); Low-grade squamous intraepithelial lesion (LSIL)

MOLECULAR TESTING FOR HPV

- Several molecular biomarkers have been recognized which roughly identify specific stages in the natural history of HPV infection and cervical cancer progression.
- They include the presence of viral proteins, viral nucleic acids (DNA and RNA) or alteration of cellular factors induced by viral oncoproteins.
- There are a number of tests that have been used for the detection of molecular biomarkers of HPV (Table 58.3).

Table 58.3: Classification of HPV tests

Type of test	*Name of test*
Nucleic acid hybridization assays	Southern blot *In-situ* hybridization
Non-nucleic acid signal amplification assays	Hybrid capture 2® Cervista® HPV HR
Nucleic acid amplification assays	Roche Cobas® 4800 Aptima® HPV HR Papillocheck® BD onclarity® Next generation sequencing

NUCLEIC ACID HYBRIDIZATION ASSAY

- This method uses radioactive nucleic acid hybridization assays to detect HPV infection.
- But this method has some disadvantages like—low sensitivity, need for a larger amount of purified DNA and it is time-consuming procedure.
- Therefore not used nowadays.

SIGNAL AMPLIFICATION ASSAYS

Hybrid Capture 2 (HC2)

- It is a nucleic acid hybridization assay with signal amplification that utilizes microplate chemiluminescent detection.
- It is a USFDA-approved quantitative assay and current gold standard for HPV detection.
- It detects 14 HPV high-risk types but does not distinguish specific genotype.
- Sample for hybrid capture 2.
- The specimen for HC2 is collected by using a cervical broom in ThinPrep PreservCyt® solution.
- At least 4 mL of sample is required and it can be stored at room temperature for up to 2 weeks.

Steps of Hybrid Capture 2

- First denature the specimen.
- Then hybridize with HPV RNA probe.
- Then capture hybrid using monoclonal antibody or microtiter plates.
- After that a secondary antibody conjugated with alkaline phosphatase is added.

- This alkaline phosphatase splits the chemiluminescent substrate to produce light which can be detected as a signal (Fig. 58.3).

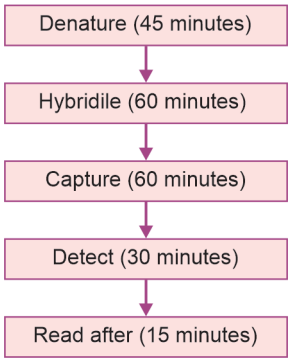

Fig. 58.3: Steps of hybrid capture 2

Advantages

- It has high sensitivity (96%) and lower false-positive rates.
- It is reproducible as a screening assay.

Limitations

- It distinguishes between low-risk and high-risk groups but does not permit the identification of specific HPV genotype.
- It has less specificity than PCR.

Cervista HPV HR

- It is also USFDA a approved and qualitative assay.
- It also detects 14 HPV high-risk types.
- It is based on the same principle as HC2.
- The sample for CERVISTA can be taken in either ThinPrep or SurePath solution.
- In comparison to HC2, the Cervista assay demonstrates 89% sensitivity in the detection of CIN3 and 98% sensitivity in CIN2 detection.

NUCLEIC ACID AMPLIFICATION ASSAYS

Roche Cobas 4800 HPV Assay

- It is also USFDA-approved method.
- Its principle is based on PCR technique.
- It also detects 14 HPV high-risk types but it specifically detects HPV 16 and HPV 18.
- This method is also clinically validated for ASC-US triage.
- The sample for CERVISTA can be taken in either ThinPrep or SurePath solution.
- ROCHE COBAS kits are available for testing.

Advantages

- It is a fully automated method.
- It has a high throughput capacity, that is approximately 280 samples run in one day.
- It specifically detects HPV 16 and 18.

Limitations

- It is relatively expensive and requires well-established laboratory settings.
- It has low sensitivity and poor predictive value.
- It comes positive in regressive lesions also.

Aptima HPV HR

- It is USFDA-approved transcription-mediated amplification-based assay to detect E6 and E7 mRNA of 14 high-risk HPV types.
- HPV mRNA is an indicator of viral gene expression.

- It can be carried out on liquid-based cytology samples, similar to DNA testing, thus requiring no significant change in screening infrastructure.
- In this technique, reverse transcriptase (RT) is used to generate a DNA copy of the target mRNA sequence containing a promoter sequence for RNA polymerase.
- One primer contains a T7 promoter sequence for RNA polymerase that hybridizes to the target RNA (Fig. 58.4).
- RT creates a cDNA of the target RNA by extension from the 3′ end of the promoter-primer.
- The RNA of the resulting RNA–DNA duplex is degraded by the RNAase H activities of the RT.
- A second primer then binds to the cDNA containing the promoter sequence from the T7 promoter-primer.
- After that RNA polymerase produces multiple copies of RNA amplicon (amplification) from the DNA copy template which get detected.

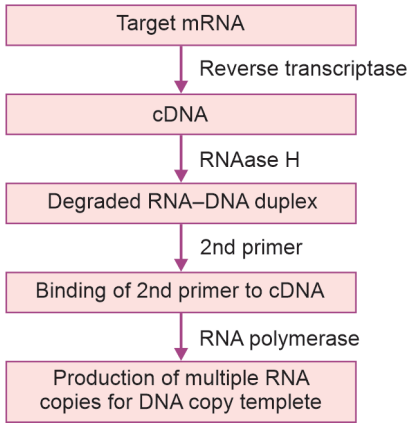

Fig. 58.4: Steps of transcription-mediated amplification

HPV Oncotect® E6, E7 mRNA KIT

- It measures both the number of transforming cells and the quantity of E6, E7 mRNA in each cell.
- Its sensitivity is equivalent to HR HPV DNA tests (95% detection of ≥ CIN2) with increase in specificity.

Advantages of RNA Detecting Methods

- These methods show a fairly good correlation between the degree of positivity and the severity of the lesion.
- More powerful methods than DNA testing for predicting the risk of progression.
- They have better positive predictive value and specificity than HPV DNA test.
- There is less number of colposcopic referrals by these methods.

Limitations

- More costly and require a well-established laboratory setting.
- They have limited role in resource-poor countries like India.

BD Onclarity Assay

- It is the most recent USFDA-approved method.
- It is also based on DNA amplification by RT-PCR, targeting E6 and E7 DNA.
- It also detects 14 HPV high-risk types along with specific detection of 16,18 and 45.

Advantages

- The sample is collected in SurePath solution. The sensitivity of this method for CIN-2/3 is equivalent to HC2.
- It has excellent specificity for CIN-2 detection and does not react with low-risk HPV types.

Limitations

- It cannot be used to evaluate non-cervical specimens for HPV detection.
- It cannot be used in women with prior ablative and excisional therapy or who are pregnant.

Next-Generation Sequencing (NGS)

- NGS has emerged as a powerful tool for HPV genotyping, with the advantage of processing multiple samples at a time.
- NGS is a technology based on massively parallel sequencing or "deep sequencing" of nucleic acid sequences.
- Nucleic acid sequences are fragmented with each fragment being amplified and sequenced multiple times, providing a depth of information that can deliver accurate data at the nucleotide level.
- NGS can sequence the entire genome of HPV or be limited to specific areas of interest.

Steps of Next-Generation Sequencing

- The whole NGS process requires several steps, which include initial sample identification, processing (nucleic acid extraction), viral enrichment (optional), library preparation, sequencing, and bioinformatic analysis of the raw data (Fig. 58.5).

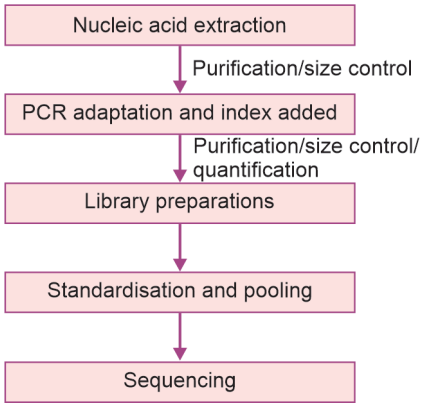

Fig. 58.5: Steps of NGS

Advantages

- Highly sensitive and specific method.
- It has the ability to detect types at low copy number (even within multiple infections).
- Novel types that are distantly related to primers/probes which may escape detection using standard molecular approaches are also detected.

Limitations

- More costly to be used as a cervical cancer screening tool.
- Time taken by NGS (1–2 days) is long as compared to PCR (3-4 hours) based methods (as it amplifies the whole genome).

COMPARISON OF HC2 WITH OTHER METHODS (TABLE 58.4)

1. HC2 Versus Roche Cobas

- Both have the similar sensitivity but negative predictive value of COBAS is more as compared to HC2.
- COBAS has more sensitivity for samples collected in SurePath solution while similar sensitivity for samples collected in ThinPrep solution.

2. HC2 Versus Aptima

- Both are used for ASC-US triage to detect underlying high-grade neoplasia.
- Both have the same sensitivity.
- The specificity of APTIMA is more for CIN-2 lesions in ASC-US triage as compared to HC2.

3. HC2 Versus BD Onclarity

- Both have similar sensitivity.
- No cross-reactivity with low-risk HPV types in BD on clarity, therefore less chances of false-negative result.

4. HC2 Versus NGS

- Has high sensitivity and specificity.
- Detects HPV DNA at low copy number.
- But not FDA-approved and more time-consuming.

Table 58.4: Comparison of various methods for HPV testing

Test	*Hybrid Capture 2*	*Cervista*	*Roche Cobas*	*Aptima*	*BD Onclarity*
Year USFDA approved for HPV testing	2001	2009	2011	2011	2018
Year approved for primary screening	2012	NA	2014	NA	2018
Sample	ThinPrep only	NA	ThinPrep and SurePath	NA	SurePath only
Method	DNA (Non-PCR based) signal amplification: full genome probe	DNA (Non-PCR based) signal amplification: L1, E6 and E7	DNA (PCR based) target amplification: L1Gene target	mRNA (PCR based) target Amplification: E6/E7 target	DNA (PCR based) Target Amplification: E6/E7 gene target
Genotypes detected	14 high-risk HPV types	13 high-risk HPV types	14 high-risk HPV types with genotyping of 16 and 18	14 high-risk HPV types; genotyping as separate test (16,18/45)	14 high-risk HPV types; simultaneous, discrete identification of 16,18 and 45
Clinical trial	ASC-US/LSIL Triage study, 2006 CAP	Cervista HPV HR	ATHENA	CLEAR trial	Onclarity trial (baseline)
Clinical validation	Extensive	Limited	Limited	Limited	Limited
Sensitivity for CIN2/3	96%	89%	94.9%	94.1%	80%
Specificity for CIN2/3	25%	–	33%	42%	52%

POINTS TO REMEMBER

- Cervical cancer is the second most common cause of mortality in India.
- Screening and early detection plays an important role in decreasing mortality.
- Cytology alone has a sensitivity of only 51% in detecting early lesions.
- Cytology + HPV testing- Increase sensitivity by 35%.
- Hybrid Capture 2 is still the gold standard and is widely in used.
- ROCHE COBAS is replacing the HC-2 these days because of more specificity for 16 and 18 types.
- NGS is an emerging technique nowadays for HPV testing.

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Cytological Approach to Mediastinal Lesions

59

Harsh Batra

INTRODUCTION

- The mediastinum is an anatomical space bounded laterally by the pleura of right and left lung, anteriorly by the sternum and posteriorly by the thoracic vertebra, superiorly by thoracic inlet and inferiorly by the diaphragm.
- The mediastinum is divided into anterior–posterior and middle mediastinum with each having specific contents (Table 59.1).

Table 59.1: Contents of mediastinum

Anterior mediastinum	*Middle mediastinum*	*Posterior mediastinum*
Thymus	Pericardium	Esophagus
Aortic arch	Heart	Vagus nerve
Superior vena cava	Great vessels	Sympathetic chain
Lymph node	Trachea	Thoracic duct
Parathyroid	Bronchi	Azygous and hemiazygous vein
Ectopic thyroid	Phrenic nerve	Paravertebral lymph node
	Hilar lymph node	

- The mediastinum shows a wide variety of lesions (both neoplastic and non-neoplastic) with variable cytomorphology and this may lead to diagnostic problems (Table 59.2).

Table 59.2: Mediastinal lesions and their distribution

Anterior	*Superior*	*Middle*	*Posterior*
Thymic tumors Germ cell tumors Thyroid tumors Parathyroid tumors Lymphoma Paraganglioma Hemangioma Lipoma	Thymic tumors Thyroid tumors Lymphoma Parathyroid tumors	Pericardial cyst Bronchial cyst Lymphoma	Neurogenic tumors Schwannoma Neurofibroma Ganglioneuroma MPNST Paraganglioma Neuroblastoma Gastrointestinal cyst Bronchogenic cyst

- The diagnosis of the mediastinal tumors by fine needle aspiration cytology (FNAC) requires a combination of clinical, radiological, biochemical, and cytomorphological information.

CLINICAL HISTORY

- The first step in diagnosing mediastinal lesions is a correct and comprehensive clinical history.
- since majority of the lesions are asymptomatic and found on regular screening.
- The common complaints of the patients are cough, chest pain, dyspnea, hemoptysis and superior vena caval syndrome due to the pressure effect on great vessels.
- Young patients may have germ cell tumors, thymoma and lymphoma. Older patient often have metastatic tumors.
- Clinical history of myasthenia gravis is usually associated with thymoma.
- Past history of malignancy may indicate metastasis.
- Presence of generalized lymphadenopathy or hepatosplenomegaly may indicate lymphoma or leukemic deposits.

RADIOLOGICAL CORRELATION

- The next step is radiological correlation.
- The delineation of mediastinum on the basis of anatomical landmarks acts as a helpful information as radiological reports can localize the lesion and can narrow down the list of differential diagnoses.

FNAC Techniques

- **CT-guided FNAC:** This can help in locating small lesions in thoracic inlet, hilum and middle mediastinum via supraclavicular, suprasternal and parasternal approaches.
- **USG-guided FNAC via percutaneous route:** It provides both spatial orientation and real-time monitoring without radiation exposure.
- FNA samples can be obtained via bronchoscopic and endoscopic techniques also.

CYTOMORPHOLOGY

- It is important to identify the basic cellular pattern in the smears.
- The major patterns are as follows:
  - Round to oval cells
  - Spindle cells
  - Pleomorphic pattern
  - Not otherwise specified
- The diagnostic approach to these patterns is shown in Figs 59.1 to 59.3.

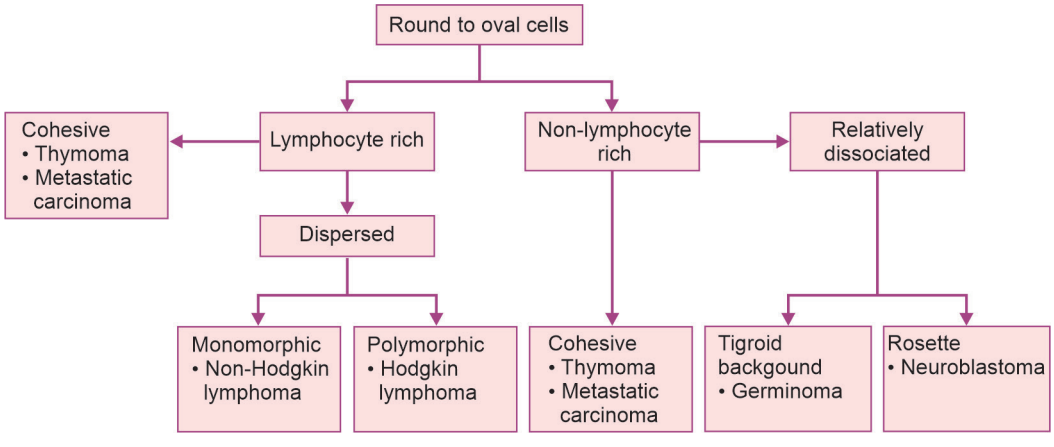

Fig. 59.1: Cytomorphological approach to cellular pattern with round to oval cells.

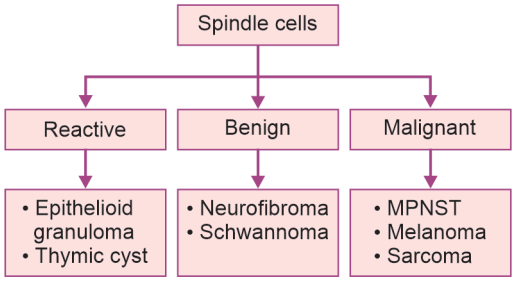

Fig. 59.2: Cytomorphological approach to cellular pattern with spindle cells

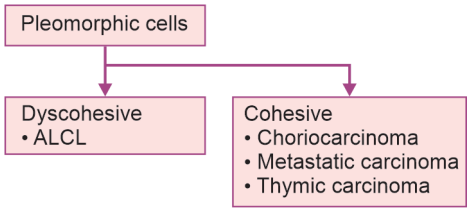

Fig. 59.3: Cytomorphological approach to cellular pattern with pleomorphic cells

ANTERIOR MEDIASTINAL LESIONS

- Certain lesions present more often in a particular age group and hence age can help in narrowing down the differentials for a lesion (Table 59.3).

Table 59.3: Age-wise distribution of anterior mediastinal lesions

10–30 years	*30–50 years*	*50–60 years*
Germ cell tumors	Seminoma, lymphomas	Thymoma

- Thyroid tumors can be seen in any age group.
- Patients may present with any of the following:
  - Asymptomatic mass in the mediastinum.
  - Cough, dyspnea, chest pain, dysphagia, hoarseness.
  - Paraneoplastic manifestations such as myasthenia gravis, red cell aplasia and acquired hypogammaglobulinemia.
  - CT scan shows homogeneous solid mass with soft tissue attenuation and well-demarcated borders.

Thymoma

- Thymoma is the neoplasm that develops from common thymic epithelial stem cell of endodermal origin.
- The patients of thymoma usually complain of local pressure symptoms of the mass such as pain, tachycardia, superior vena caval syndrome and respiratory distress.
- It is often associated with myasthenia gravis.
- According to recent World Health Organization (WHO) classification thymoma is classified as:
  - Type A
    - Bland oval to spindled cells
    - Rare or no thymocytes
    - Various patterns described include hemangiopericytoma-like vascular pattern, rosettes/pseudorosettes, microcystic changes.
  - Atypical A
    Same as A along with focal
    - ≥ 4 mitoses/10 high power fields or
    - Coagulative tumor necrosis or
    - Hypercellularity

- Type AB
  - Morphology of A+B1 or A+B2, components can be intermingled or separated
- Type B1
  - Medullary islands (Hassall corpuscle-like elements, paler areas), scattered neoplastic cells (<3 contiguous epithelial cells)
- Type B2
  - Mixed neoplastic cells and thymocytes, medullary islands occasionally present
- Type B3
  - Neoplastic cells
  - Rare or no thymocytes

- It is not recommended to do subtyping on small biopsies because of tumor heterogeneity.
- Cytology smears show the dual population of multiple clusters of epithelial cells in the background of lymphoid cells.
- The epithelial cells in thymoma are arranged singly or as large clusters.
- The cells have moderate cytoplasm with indistinct cell margins.
- The nuclei are vesicular with pale homogeneous chromatin and single prominent nucleoli.
- The background of the smear shows single naked cells with stripped- off cytoplasm.
- Spindle cell variant of thymoma shows oval to elongated spindle cells in small clusters.
- The cells are arranged in small fascicles that are better appreciated in cell block preparation.
- Lymphocyte-rich thymoma in FNAC shows predominantly lymphocytes and scanty epithelial cells.

Immunocytochemistry

- Epithelial cells of thymoma are positive for cytokeratin (CK), epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA).
- Thymic tumor cells are strongly positive for AE1-defined acidic CKs and negative for AE3-defined basic CKs.
- The epithelial cells also show focal positivity for CD20, the B cell marker.

Differential Diagnosis

- Non-Hodgkin's lymphoma: Type B1 thymoma contains lymphoid cells of immature thymic type. These may be selectively sampled and may give a false impression of lymphoma. On flow cytometry, maturing T cell phenotype is characteristic of thymoma.
- Thymic carcinoma
- Thymic hyperplasia
- Mesothelioma: Monolayered sheets with intercellular windows are seen.

Thymic Carcinoma

- It is a rare malignancy of the thymus and predominantly involves adults.
- Unique features include:
  - No association with myasthenia gravis
  - Rarely associated with autoimmune diseases

- Positive for CD5, CD70 and CD117
- Almost always invasive
- Mostly unresectable tumor

- Cytology:
  - Malignant epithelial cells with irregular configuration have abundant eosinophilic cytoplasm, large nuclei, coarsely clumped chromatin and prominent nucleoli.

Immunocytochemistry

Shows positivity for CD5, CD70 and CD117.

Differential Diagnosis

- Metastatic carcinoma: Anaplastic carcinoma from other sites is not distinguishable from primary thymic carcinoma on cytomorphology alone. Ancillary tests are necessary to confirm the diagnosis.
- CD5 positivity supports the diagnosis of primary thymic carcinoma.

Lymphoma

- These include Hodgkin's and non-Hodgkin's lymphoma.
- Nodular sclerosing type is common amongst Hodgkin's lymphomas and mediastinum is usually a common site.

Clinical Features

- Young adult with a bulky mediastinal mass
- Supraclavicular lymphadenopathy
- Respiratory difficulties due to mediastinal mass
- Superior vena caval obstruction
- Pleural effusion.

Cytology

a. Hodgkin's lymphoma: Polymorphous cell population comprising eosinophils, plasma cells and RS cells. RS cells are positive for CD30.

b. Non-Hodgkin's lymphoma:

i. *T-lymphoblastic lymphoma:*
   - Relatively uniform cell population with a high mitotic rate
   - Intermediate sized nuclei
   - Variable number of convoluted indented nuclei
   - Dense finely granular chromatin
   - Scanty, pale fragile cytoplasm
   - Immunophenotype: TdT, cy CD3 and CD7.

ii. *Diffuse large B cell lymphoma*
   - Predominance of large centroblastic lymphoid cells with pale nuclei, scant cytoplasm and multiple often peripheral nucleoli.
   - Immunophenotype: CD19, CD20, CD79a.

Germ Cell Tumors

- Mediastinum is the most frequent site of origin for extragonadal germ cell tumor.
- However, metastatic tumors are more common than the primary. So search for the primary in gonads should be done first.
- Germ cell tumors include:
  - Teratoma
  - Germinoma (seminoma/dysgerminoma)
  - Embryonal cell carcinoma
  - Yolk sac tumor
  - Choriocarcinoma
- Clinical features and radiology:
  - Shortness of breath, chest pain, cough.
  - CT scan: Well-defined unilocular or multilocular cystic lesion containing fluid, soft tissue or fat attenuation.
  - MRI: Heterogeneous signal intensity representing various internal elements.

Mature teratoma

- It constitutes 7–9% of all mediastinal germ cell tumors.
- Cytological features include mature squamous cells, anucleate squames, keratin flakes, glandular cells and presence of neural elements.

Immature Teratoma

- It is a rare mediastinal tumor.
- On cytology, smears show round to spindled with fine granular chromatin, indistinct cytoplasm arranged in rosette and tubules (immature neuroectodermal elements) in fibrillary background.

Seminoma

- Another rare tumor comprising 8% of extragonadal germ cell tumors.
- Cytology: Cell-rich smear with dispersed cells with a little tendency for clustering.
- Highly fragile cytoplasm and nuclei (tigroid background).
- Large rounded vesicular nuclei, distinct nucleoli and irregular chromatin with the presence of plasma cells and lymphocytes.

Embryonal Carcinoma

- Comprises about 12% germ cell tumors of mediastinum.
- Cytology: Cell-rich smear with frequent three-dimensional clusters.
- Large vesicular nuclei and prominent nucleoli
- Basophilic to amphophilic sometimes pale, vacuolated cytoplasm.

Endodermal Sinus Tumor

- A rare tumor occurring in infants and young children.
- Cytology shows small clusters, papillae and discrete malignant cells which are moderately pleomorphic having vacuolated cytoplasm.
- Occasional intracytoplasmic and extracytoplasmic small round pink globules are also seen.

Thyroid and Parathyroid Lesions

- The lesions include goiter, thyroid cancer and parathyroid neoplasms.
- Clinical features and radiology:
  a. Shortness of breath, dyspnea, palpitation, cough.
  b. CT scan: Enlarged thyroid gland/ extension of substernal goiter.
  c. USG:
    i. *Benign lesion:* Iso-hyperechoic with peripheral/coarse calcification
    ii. *Malignant lesion:* Hypoechoic solid, microcalcification.

Colloid Goiter

Cytological features

- Thick or thin colloid.
- Follicular cells in monolayered sheets, poorly cohesive clusters and single cells.
- Hyperplastic and involutional follicular cells.
- Fragile cytoplasm, bare nuclei.
- Foam cells.

Papillary Thyroid Carcinoma

Cytological features

- Multilayered complex papillary arrangement of follicular cells.
- Sheet of follicular cells that are enlarged, ovoid with pale nuclei and granular chromatin.
- Nuclear crowding and overlapping.
- Nuclear inclusions.
- Nuclear grooves.

Follicular Carcinoma

Cytological features

- Moderate to high cellularity.
- Colloid-free background.
- Prominent microfollicular pattern.
- Nuclear crowding and overlapping.

Hurthle Cell Neoplasm

Cytological features

- Sheets of oxyphil cells
- Oxyphil (Hurthle) cells are large, polygonal cells with oval nuclei and abundant granular cytoplasm
- Nuclei are eccentrically placed
- They are also seen in nodular goiter, Graves' disease and Hashimoto thyroiditis.

Medullary Carcinoma Thyroid

Cytological features

- Cellular smear with dispersed cells.
- Plasmacytoid, small cell and spindle cell pattern.
- Stippled nuclear chromatin.
- Amorphous pink/violet background material (amyloid).
- A few scattered cells with coarse red cytoplasmic granularity.

Parathyroid Neoplasms

Parathyroid lesions constitute adenoma in 80–85% of the patients followed by hyperplasia in 6–15% of the patients and carcinoma in <1% of the patients.

Cytological features

- Nuclei are round and have a coarsely granular chromatin pattern.
- Nucleoli are small or prominent.
- Cytoplasm is moderately abundant and granular.
- Isolated cells and naked nuclei can be present.
- Focal nuclear pleomorphism is seen, but it is not prominent.
- Colloid is absent.
- Hypercalcemia usually acts as a clinching point to differentiate parathyroid adenoma from follicular lesion of thyroid.

MIDDLE MEDIASTINAL LESIONS

These include bronchogenic cysts, neuroendocrine tumor, squamous cell carcinoma, adenocarcinoma, lymphomas, vascular tumors and paraganglioma.

Bronchogenic Cyst

- These are most commonly found in young adults.
- Symptoms are the result of compression, e.g. difficulty in breathing or swallowing, cough and chest pain.
- Chest X-ray: Smooth density in front of the trachea or at the carina.
- CT scan: Smooth or oval mass with homogeneous attenuation with no enhancement of cyst contents and no infiltration of adjacent structures.

Neuroendocrine Tumor

Cytological features

- Small or medium-sized cells with a little or no cytoplasm.
- Dispersed cell presentation, some clusters including some tight groups.
- Nuclear molding and engulfment.
- Uniform finely or coarsely granular nuclear chromatin, small nucleoli.

Paraganglioma

Cytological features

- Neoplastic cells singly and loosely clustered, often forming a vaguely follicular pattern.
- Abundant pale cytoplasm with indistinct cell border.

- Fine red cytoplasmic granulation.
- Nuclei round to spindle with granular or speckled chromatin.
- Neuroendocrine markers on cell block for confirmation.
- Vascular tumor: Hemangiomas in the mediastinum, although asymptomatic but may sometimes present with hypertension, sweating, tachycardia, headache, tremor.
- On CT, a hypervascular lesion is seen.
- Cytological features: A few strands of endothelial cells with pale spindly nuclei.
- Epithelioid pattern of gland-like cluster of malignant cells with round nuclei.
- Cell block shows CD34 positive cells.
- Lesions in the posterior mediastinum include schwannoma, neurofibroma, malignant peripheral nerve sheath tumor (MPNST), undifferentiated pleomorphic sarcoma and synovial sarcoma.

Clinical Features and Radiological Features

- They are usually asymptomatic mediastinal masses.
- When large, these present with chest tightness and cough.
- CT scan: Helps determine the location of the mass and usually presents as a well-circumscribed mass (benign).
- A sudden increase in size and presence of neurological symptoms may suggest malignant transformation.

Schwanomma

Cytological features

- Tissue fragments of cohesive cells.
- Mainly dispersed cells and only small tissue fragments in a myxoid background.
- Fibrillary appearance of the intercellular stroma is the most typical feature.
- Nuclear palisading and Verocay body is seen.
- Nuclei are long and slender with pointed ends, some are comma-shaped and bent like a fish-hook.

Neurofibroma

Cytological features

Loosely arranged cluster of wavy spindle cells separated by amorphous myxoid stroma. The spindle cells contain finely granular chromatin.

Malignant Peripheral Nerve Sheath Tumor (MPNST)

Clinical Features and Radiological Features

- Dull chest pain, fatigue
- Breathing difficulties
- Weakness of lower limb
- X-ray: Homogeneous soft tissue opacity in retrocardiac lesion
- CT scan: Well-defined lobulated soft tissue enhancing mass

Cytological features

- Highly pleomorphic cells having epithelioid appearance
- High-grade tumors, smear is highly cellular and the cells have elongated spindly atypical nuclei

Differential diagnosis

- Spindle cell thymoma, carcinoid tumor, connective tissue neoplasm and lymphoma
- Diagnosis of malignancy and specific typing of MPNST is extremely difficult on aspirated material.
- Some benign lesions are extremely pleomorphic and cellular.
- If cell block with ample tumor is available, S-100 immunostaining may help. With benign nerve sheath tumor, staining is diffuse and intense, whereas their malignant counterparts are weak/focal/negative.

Neuroblastoma

Cytological features

- Hypercellular smear
- Individually scattered anaplastic cells having scant cytoplasm, round nuclei, high N: C ratio, granular chromatin, inconspicuous nuclei.
- Also seen are clusters of cells arranged in Homer Wright rosettes containing central neuropil.

Undifferentiated Pleomorphic Sarcoma

Cytological features

- Cellular smear, single scattered cells and cohesive cluster.
- Atypical spindle cells with elongated nuclei, moderate cytoplasm, marked nuclear pleomorphism, coarse chromatin and often prominent nucleoli.
- Multinucleated tumor giant cells and bizarre nuclei.

Synovial Sarcoma

Cytological features

- Highly cellular smear with a typical appearance of a mixture of tissue fragments and dispersed cells.
- Cells are small to medium.
- Scant cytoplasm, rounded or ovoid nuclei with finely granular bland chromatin and nucleoli are inconspicuous.
- Poorly differentiated synovial sarcoma is difficult to differ from Ewing's sarcoma.

Diagnostic Pitfalls

- Morphological heterogeneity within the lesions.
- Stromal sclerosis resulting in low cellularity.

POINTS TO REMEMBER

- Mediastinum is involved in various primary and metastatic tumors.
- Tumors in this region have variable therapeutic management and prognosis, hence it is important to reach the correct diagnosis by FNAC.
- The reported sensitivity and specificity of diagnosis of mediastinal lesions by FNAC is 88% and 82% respectively.

- Mediastinum is surrounded by many vital structures such as heart and great vessels.
- Therefore, precautions are needed to perform FNAC.
- A team of experts of radiologist, cytologist, physician and surgeon is required to perform FNAC.
- Usually USG-guided FNAC is performed.
- Initial assessment of clinical features and radiological data narrows down probable diagnostic categories.
- Aspirated material should be collected for cell block, flow cytometry and microbial culture. The stepwise algorithmic approach provides a definite diagnosis in most cases.

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60 Circulating Tumor Cells (CTC): Overview and Opportunities in Cytology

Epsita Das

INTRODUCTION

- Circulating tumor cell (CTC) was first described by Thomas Ashworth in 1869.
- It is derived from tumors as an early step and is a major contributor in tumor metastasis.
- It has a prognostic significance in cancer detection.
- These tumor cells are transient in blood with a half-life of 1 to 2.4 hours.
- However, they are low in abundance in most cancer patients (<10 cells per milliliter).
- Cell-free DNA (cfDNA)—how is it different?
- Comparison between the two is shown in Table 60.1.
- DNA can be released by both benign and malignant lesions into the blood through apoptosis and necrosis.
- They are elevated in a variety of pathological processes.
- Another origin of cfDNA is cells that are actively secreting DNA.

Table 60.1 Comparison between circulating tumor cells (CTCs) and cell-free DNA (cfDNA)

Comparison	*CTCs*	*cfDNA*
Definition	Tumor cells released from primary tumors/metastatic sites in blood	High concentration of cell-free DNA in tumor patients
Information type providing analysis	DNA, RNA and protein	DNA
Common detection technology	Cell search method	Conventional approach (karyotype analysis) Molecular cytogenetic methods (CGH, FISH), microarray-based CGH, SNPs, NGS, PCR methods
As diagnostic biomarkers	Contributes to judgment of benign and malignant diseases	Diagnosis of molecular subtypes
Roles in targeted therapy	Baseline CTCs count used to predict and monitor efficacy of targeted therapies	Monitor mutation status to guide selection of targeted drugs

NEED FOR CTC KNOWLEDGE

- 90% of cancer deaths occur due to metastasis (according to CDC).
- Functional and molecular characterization of CTCs helps in the in-depth knowledge of the lethal disease.
- Knowledge of CTCs helps in cancer monitoring and designing targeted cancer treatments.

PROBLEMS IN CYTOLOGY

- Smear cellularity depends on appropriate sampling techniques during FNA.
- Cytology deals with paucicellular liquid specimens (e.g. CSF).
- Definite diagnosis and characterization of tumor is usually not possible without cell block preparation and ICC.
- Many overlapping features are shared by reactive and malignant lesions.

CTC BIOLOGY

- Cascade of intravasation-translocation-extravasation-colonization.
- Constraints to distant metastasis:
  1. Tight junctions, desmosomes, hemidesmosomes (between cancer cells)
  2. Complex stroma composed of extracellular matrix (ECM), blood vessels, BM
- Metastasis: Through EMT (Epithelial-mesenchymal transition) or non-EMT mediated entry.

Non-EMT Mediated Entry

1. Centrosome amplification: (Seen in transgenic mouse model).
2. Accidental CTCs: Due to surgery.

Existence of CTC in Blood After Metastasis

- Major under go apoptosis. Minority are camouflaged cells.
- This is because of a lack of correct cellular environment.
- Adaptation to the circulatory environment, innate immunity, oxidative stress and evasion of immune defense by increasing various surface proteins (CD 47, PDL1, VCAM 1) play a role.

Existence of Heterogeneous Forms of CTC

- CTC in blood: As apoptotic or viable cells.
- Subpopulation: Single-cell or clusters.
- Harbor variation in size depending on tissue derived from 3 distinct CTC subpopulations detected in prostate cancer patients' blood:
  1. Large nuclear (>14.99 μm)
  2. Small nuclear (8.54 –14.99 μm) in metastatic disease
  3. Very small nuclear (vsn) (<8.54 μm) in visceral metastasis (lungs, liver).
- Increased CTCs indicate disease progression.
- Majority of patients with advanced disease conditions show single CTCs.
- Minority with advanced disease: Harbor single CTCs and clusters simultaneously.

Terminology in CTC Isolation Methods

- **CTC enumeration:** Simply count the number of CTCs
- **CTC enrichment:** Enhances the proportion of CTCs in a specimen as compared with the number of non-tumor cells predominantly (white blood cells) (low number, fragility and heterogeneous nature of CTCs in patient's blood)
- **Enrichment techniques:** Through *ex vivo*/*in vivo*/newer devices methods (Fig. 60.1).

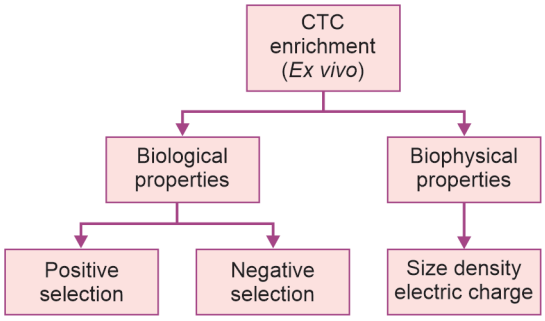

Fig. 60.1: Showing enrichment techniques of circulating tumor cells based on physical and biological properties.

Physical Property Dependent Enrichment Methods

- Epithelial-derived CTCs: Larger and stiffer compared to leukocytes (7–12 μm in diameter).
- Filtration-based devices developed to isolate these CTCs.
- Blood is filtered through pores (usually 8 μm) to trap molecules larger than the maximum pore sizes.
- Enrichment is determined by factors like blood flow rate, pore size uniformity, and membrane rigidity.

Morphologic Features of CTCs in Filtration Method

- CTCs show morphologic similarities to the tumor cells seen in cytologic preparations.
- The morphologic criteria proposed to identify CTCs include:
  - Anisonucleosis (ratio from one nucleus to another of >0.5)
  - Nuclei larger than 3 times the calibrated pore size (pore: 8 μm; nucleus >24 mm)
  - Irregular nuclei
  - Presence of 3-dimensional sheets
  - High nuclear/cytoplasmic ratio.

Density Gradient Centrifugation

- Enriches CTCs in the mononucleocyte fraction in a buoyant density taking advantage of their similarity.
- Bi-specific antibodies against antigens on leukocytes and erythrocytes have been used to increase purity.
- They form large multicellular rosettes of leukocytes and erythrocytes which can be easily removed from the mononucleocyte fraction by centrifugation.

Dielectrophoretic Field-Flow Fractionation (DEP-FFF)

- DEP-FFF combines electric force fields with hydrodynamic and sedimentation forces
- Pull tumor cells away from blood cells
- Success determined by factors like electric field frequency, electric voltage, blood flow rates and buffer formulations.
- Viability of cells enriched by DEP is greater than 97%.
- Average cell recovery of DEP only about 70%.

Biological Property Dependent Enrichment Methods (Fig. 60.2)

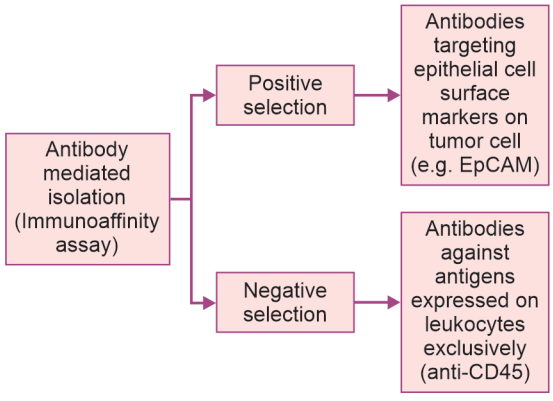
Antibody mediated isolation (Immunoaffinity assay)
Positive selection
Negative selection
Antibodies targeting epithelial cell surface markers on tumor cell (e.g. EpCAM)
Antibodies against antigens expressed on leukocytes exclusively (anti-CD45)

Fig. 60.2: Showing enrichment methods of circulating tumor cells based on biological properties

Limitation of Exclusively Involving the EpCAM Antibody

- Loss of EMT-associated CTCs.
- "Poorly differentiated and stem cell-like" cells and clusters which might represent the most aggressive CTC subpopulations.

Current Focus on Search for New CTC Surface Markers

- Actin bundling protein, Plastin 3 as they are not down-regulated by CTCs during EMT.
- Not expressed in blood cells.

Another Improvement: Cocktail of Antibodies

- Against mesenchymal or stem cell antigens (c-MET, N-cadherin, CD318, and mesenchymal stem cell antigen)
- Against tissue-specific markers (PSA for prostate cancer and mammaglobin for breast)
- Against various tumor-specific markers (HER2, MUC1, EGFR, folate-binding protein receptor, TROP-2, CA9, and CD147) has been created to enrich CTCs.

NEWER DEVICES (Fig. 60.3)

Cell Search System

- Most common method of CTCs enrichment
- Only US FDA-approved device for CTC detection in breast, prostate and colorectal cancer
- Immunobead assay using anti-EpCAM antibodies.
- The magnetic beads coated with antibodies attach to antigens.
- Subsequently removed by applying a magnetic field.
- Pure yield but longer sample processing time.

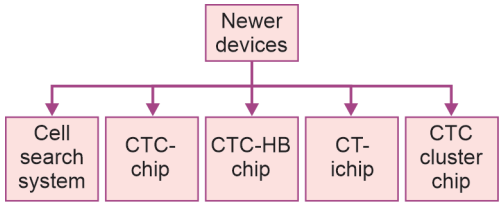

Fig. 60.3: Showing newer devices including microdevices for circulating tumor cells enrichment

MORPHOLOGIC FEATURES OF CTCS IN CELL SEARCH METHOD

- Morphologically CTCs by Cell Search Criteria should have
- Nuclear 4′,6-diamidino-2-phenylindole (DAPI) +
- Cytoplasmic keratin +
- CD45 cytoplasmic staining –
- EpCAM +
- Cell-like morphology
- Minimum size requirement.

Microdevices and Microfluidic Platforms

- Integrating microfluidics into CTC enrichment technology has been another milestone to enrich yield.
- Facilitates separation and detection of small quantities of samples.
- High sensitivity in the short time frame.
- There is use of antibody cocktails in addition to anti-EpCAM proteins.
- More precise isolation of CTCs.

CTC-CHIP

- Silicon-based micropost.
- Thousands of microposts coated with EpCAM antibody.
- Increased encounters between flowing cells and antibodies improving the enrichment efficiency of CTCs
- Disrupt CTC clusters due to the tight distances between microposts
- Prevent the passage of large cellular clusters.

HERRINGBONE(HB) CTC-CHIP

- Original CTC-CHIP developed into one with herringbone groove patterns by toner.
- Advantage: Blood flow is less streamlined and increased capture efficiency.
- More CTCs contact antibodies lining the chip's inner walls.
- Limitation: Cell viability compromised.

CTC-ICHIP

- Developed by toner group developed
- Enrichment of CTCs with either positive or negative selection.
- First size-based separation of the nucleated cells including CTCs and WBCs from RBCs, platelets and plasma.
- Nucleated cells arrayed in a single line due to inertial focusing as they traveled through specially configured curved channels.
- Inertial-focused tagged leukocytes are magnetically deflected as they travelled through channels equipped with magnets.
- Untagged CTCs are delivered at an average purity of 1% in solution
- Can be subjected to subsequent molecular characterization.

CLUSTER-CHIP

- Developed by toner group.
- To examine the true prevalence and significance of CTC clusters.

- CTC clusters isolated through specialized bifurcating traps under low-shear conditions (preserve cluster integrity)
- Clusters in 30–40% of patients with metastatic breast, prostate, or melanoma were identified.

Limitation of Ex Vivo Enrichment Techniques

- Blood used in these methods is usually less than 10 mL
- Limited volume of blood cannot represent the whole CTC population in a patient
- Decreases the reliability of CTC biopsies
- To overcome these limitations: *in vivo* enrichment of CTCs has been developed.

In Vivo CTCs Enrichment Methods (Fig. 60.4)

1. GILUPI nanodetector (1.5 L of blood passes the nanodetector coated with EpCAM antibodies in 30 minutes put in a peripheral arm vein)
2. Leukapheresis (to isolate mononuclear cells from several litres of blood and used for various applications)
   - *Advantage:* Assess a large number of CTCs compared to cell search assay (a median of 7500 CTCs per patient in non-metastatic carcinoma in leukapheresis.
   - *Disadvantage:* Invasive hence limiting its clinical use.

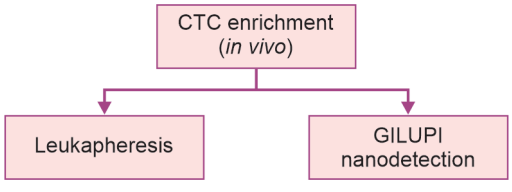

Fig. 60.4: Showing *in vivo* enrichment techniques of circulating tumor cells (CTCs)

CTC DETACHMENT FROM SURFACES

- Important challenge to effectively release CTCs from the substrate without impacting the cells
- Release methods using sheer forces-reduce viability and function of the fragile cells
- Methods for detachment of CTCs (Table 60.2)
  1. Enzymatic digestion
  2. Aptamers
  3. Polymers

Table 60.2: Different methods for circulating tumor cell detachment (CTC detachment)

Enzymatic digestion	*Aptamers*	*Polymers*
Digest extracellular matrix and detach cells	Single stranded oligonucleotides (RNA or DNA) of small molecular weight	pH responsive polymers synthesized by linking structures with weakly acidic and basic functional groups to hydrophobic base
Degrade other cells' membrane proteins and cell to cell junction proteins	Advantage-high stability resistance to harsh conditions, negligible toxicity	Once ionized, groups of similar charges cause repulsions and polymer expands and vice versa

Contd.

Table 60.2: Different methods for circulating tumor cell detachment (CTC detachment)		
Enzymatic digestion	*Aptamers*	*Polymers*
For example, trypsin, nucleases, endonucleases	Very efficient and non-invasive method	
	Changes in temp and pH alter 3D confirmation and cause aptamers to lose affinity to target molecule. Hence complimentary oligonucleotide sequence designed to bind to aptamers	

Culture, Detection and Downstream Analysis of CTCs

- Various techniques to optimize CTC culture proliferation include hypoxic conditions and non-adherent cultures
- CTCs after enrichment are detected by:
  - FISH for genome amplification
  - ICC for protein markers
  - RT-PCR/qRT-PCR for quantifying specific RNA and DNA sequences.
- After amplification cells are genotyped using:
  - Comparative genome hybridization (CGH)
  - Sanger sequencing or next-generation sequencing (NGS).
- These techniques have already been performed to:
  - Analyze genetic changes over an entire cell cycle
  - Comparing point mutations in CTCs to primary tumors.

Advantages of Liquid Biopsy Over Solid Biopsy

- No invasion inside tissue
- Helpful in cancers with osteoblastic metastasis (e.g. prostate cancer) where solid biopsy does not provide information about tumor genome evolution
- CTC can revolutionize cancer diagnosis and monitoring
- Studies showed >3 CTCs in 4 mL blood helped to differentiate local from metastatic status.

UTILITY OF CIRCULATING TUMOR CELLS BY PRIMARY TUMOR SITE

1. Breast Carcinoma

- Prognostic indicator
- Methods to assess ER, PR and (HER2) status in breast cancer CTCs have been developed.
- Patients with HER2-positive CTCs benefit from aggressive or targeted HER2 therapy, even if the primary tumor or sampled metastases are HER2-negative.

2. Prostate Carcinoma

- CTC enumeration: Prognostic indicator in prostate carcinoma.
- Scher et al found the presence of androgen receptor variant 7 (arv7) expressing CTCS with worse overall survival and predicted response to taxanes, but not androgen receptor signaling inhibitors.

- CTC-based prediction and monitoring evolves as prostate-specific antigen screening becomes less prevalent and as active surveillance of prostate carcinoma increases.

3. Colorectal Carcinoma

- Third primary tumor site for which CTC enumeration has been FDA-cleared is colorectal carcinoma.
- Testing for microsatellite instability successfully performed in CTCs.
- Suggests CTC may develop microsatellite instability when the primary tumor was previously found to be microsatellite stable.

4. Lung Carcinoma

- Small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC): CTC related to reduced overall survival.
- In NSCLC: CTCs additionally related to increased risk of lymph node metastasis.
- A study of NSCLC patients who progressed on anti-EGFR therapy, EGFR T790M mutations were identified in CTCS in 70% of patients.
- Combination of testing of CTCs and cf DNA allowed results in 35% of patients (negative or indeterminate biopsy results).
- Another study found fluctuations in CTC count useful in assessing treatment response.

5. Head and Neck Squamous Cell Carcinoma

- CTC-positive patients had increased risk of disease progression which was independent of tumor stage and nodal status.

6. Thyroid Carcinoma

- In papillary thyroid carcinoma, the combination of postsurgical serum thyroglobulin and CTCs detected by EPCAM as well as TSHR expression separated patients with metastatic disease from those without metastasis.
- CTCs: Useful substrate for BRAF V600E testing.
- Predict which patients may benefit from additional and aggressive therapy, such as radioactive iodine treatment.

7. Urothelial Carcinoma and Melanoma

- CTCs correlate with increased clinical stage in urothelial carcinoma.
- Melanoma does not express EPCAM.
- Positive selection for melanoma CTCs uses different antigens.

Enumeration of CTCs as Prognostic Indicators

- CTCs exist in the blood long before a tumor is diagnosed (potential diagnostic parameter).
- The reliability of CTCs in early cancer detection and characterization depends on the sensitivity of the method adopted and the blood volume sampled.
- Main challenge is an enumeration of all the CTC subpopulations in a blood sample
- The main methodology adopted currently is the cell search assay.

- To enrich all CTC subpopulations in a given blood sample, negative selection integrated with microfluidics (e.g. CTC-Ichip) is the ideal method.
- Direct detection of CTCs in blood samples by microscopy with automated scanning technology is an ideal alternative.

CTCs as Predictive Markers

- Genomic profiles of CTCs are comparable to primary tumors and metastases; hence they provide a snapshot of the molecular landscape of a patient's overall tumor characteristics.
- Predictive marker for guiding tailored choices of precise treatment for specific patients.
- CTCs from colorectal cancer patients showed mutations in gene APC, PIC3CA, and KRAS as detected in the matched primary tumor.
- Detection of CTCs can reveal tumor cells that present at a subclonal level in the primary tumor and metastases, which might be ignored in a tissue biopsy.

OPPORTUNITIES IN CYTOLOGY

- Cytology performs high-complex testing on liquid specimens.
- Existing workflows connect the cytology laboratory to molecular testing, including HPV testing and molecular testing for solid tumors.
- CTC technology could help confirm the identity of rare tumor cells within paucicellular specimens.
- It can be performed on a glass slide, as some technologies incorporate morphologic image interpretation on standard slides.
  1. Currently, these techniques have found success in analyzing CSF, which is often both paucicellular and very low volume.
  2. A specialized panel of immunolabels could be utilized to distinguish mesothelial cells and histiocytes from tumor cells in body cavity fluid specimens.
     - CTC technology can be used as an additional mechanism to perform multiparameter immunophenotyping of rare cells seen on routine cytology preparations:
       - Smears
       - Liquid-based preparations
       - Cytospins
       - Cell block or Paraffin-embedded tissue.
  3. Instead of expanding further specimens to create a cell block and hoping that the diagnostic cells will be abundant enough that the original slide can be reprocessed using 4- to 6-color immunolabeling to further characterize the cells in question. However, the artifacts introduced by previous staining will need to be overcome
  4. Upfront multiparameter phenotyping helpful to conserve small samples, such as fine needle aspiration or core biopsies in new cancer diagnoses.
     Example: ALK, ROS1, and PDL1 are currently performed on all lung adenocarcinomas, on serial levels of a core biopsy or cell block specimen. With multiparameter testing, tissue could be conserved for future testing of newly discovered biomarkers, reducing the need for repeat biopsies.

POINTS TO REMEMBER

- CTC technology has the potential to facilitate diagnosis and improve sample conservation of small specimens and potentially rare diagnostic cells in cytology.
- Although currently performed in a research setting, CTC testing can facilitate much of the work we perform daily in cytology as we evaluate liquid and tissue specimens.
- Appropriate incorporation of CTC technology into clinical practice requires us to understand its capabilities and mechanisms of integrating this powerful testing modality into pathology practice.

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Index

A

Aplastic anaemia 243
  diagnostic criteria 245
Approach of immunohistochemical markers in liver tumor 465, 467
Autoimmune hepatitis 156
  diagnostic criteria 156
  overlap syndromes 159
Autoimmune lymphoproliferative syndrome (ALPS) 170
Automated cervical screening techniques 102–105

B

Banff classification 535–539
Barrett's esophagus 183
  definition 183
  histological categories 186
Brachyury 522, 523

C

CAR T cell therapy 50
Cell block preparation methods 547–550
Cervista HPV HR 107
Circulating tumour cells (CTC) 340
  methods for identification 342–347
Comparison of various methods for HPV testing 565
Connexins 55
  functions 58–59
  molecular structure 55–57
  role in tumour biology 59–61
Cord blood 311
  applications 311
  testing 311
Cutaneous pseudolymphoma 385
  diagnostic approach 385

D

Dendritic cells 12
  migration 12–16
  origin 12
DNA microarray
  applications 92–94
  types 91–92

E

Epithelial mesenchymal transition 6
  historical background 6
  induction 8
  signalling pathways 7(t)
  transcription factors 9–10

F

Flow cytometry findings in various types of AML 252

G

GATA3 417
  role in various tumours 417–419
Gestational trophoblastic diseases 491–498
Gleason grade grouping 373–374(t)
Guaiac faecal occult blood test 121–122

H

Hemosiderotic fibrolipomatous tumour 111–112
Hepatitis E 417
  molecular testing 325
Hepatocellular adenoma 421
  molecular pathological classification 422–424
Hepatocyte differentiation markers 464
Hereditary renal cell tumours 140(t)
HIV associated nephropathy (HIVAN) 445, 448
  classic 448
  pathogenesis 447
HLRCC associated renal cell carcinoma 145
Hybrid capture 2 106

I

IDH mutations in glioma 196
IHC markers used in the differential diagnosis of thyroid neoplasms 457–459
IHC panel for distinction of hepatocellular carcinoma from metastatic adenocarcinoma 465(t)
Immune reconstitution inflammatory syndrome (IRIS) 19, 450
  diagnostic criteria 21
  malignancy 27–28
  paradoxical 23
  pathogenesis 22
Immunotherapy 285
  types 285
Inherited platelet disorders 287
  classification 268(t)
IPMN 480
  classification 481–482
  invasive carcinoma arising from 483

L

Laboratory accreditation 331, 333
  NABL 333
  process 337–338
Lung carcinoma 509
  molecular markers 509–512

M

Mesothelial cells 501
  adenocarcinoma vs mesothelioma 502
  benign vs malignant 501
Microparticles
  association with diseases 40–41
  functions 40
  generation 38
MIT family translocation carcinomas 145–146
Molecular pathology of AML 232–235
Multiplex immunohistochemistry 477

P

Papillary lesions of breast 525
  WHO 2019 classification 525
PD-1/PD-L1 74
  immune resistance 74
Post-transplant lymphoproliferative disorder (PTLD) 222
  classification 222(t)
Pseudomyogenic haemangioendothelioma 110–111

S

Serous tubal intraepithelial carcinoma (STIC) 133–134
Succinate dehydrogenase deficient renal cell carcinoma 146

T

Targeted therapy in myeloproliferative neoplasms 295
Tattoo reactions 79

V

Vesiculobullous skin disorders 353
  histopathological approach 355(t)

W

WHO classification of endometrial stromal tumours (2020) 407
WHO classification of pancreatic neuroendocrine tumours (2017) 201(t)
WHO classification of penile carcinoma 212
WHO classification of renal tumours (2016) 141–142
WHO/EORTC (2018) classification of cutaneous lymphomas 375–376
WHO/ISUP grading of prostatic carcinoma 370–372
WHO/ISUP grading of renal cell carcinoma 143

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